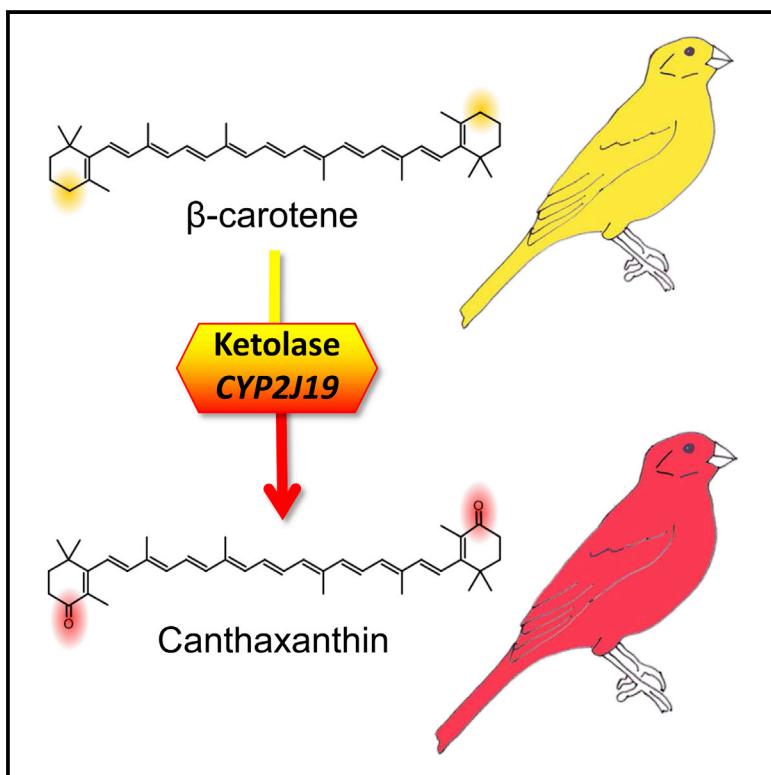


## Graphical Abstract



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## In Brief

To produce red coloration of bills and feathers, birds convert yellow dietary carotenoids to red ketocarotenoids via the action of an unknown enzyme. Lopes et al. use whole-genome sequencing of yellow and red canaries to implicate *CYP2J19* as the ketolase that catalyzes this conversion.

## Highlights

- Two genomic regions are required for red ketocarotenoid-based coloration in canaries
- The first region contains *CYP2J19*, a cytochrome P450 family member
- CYP2J19* is implicated as the ketolase required for ketocarotenoid formation in birds
- The second region maps to a cluster of genes involved in epidermal differentiation

# Genetic Basis for Red Coloration in Birds

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## SUMMARY

The yellow and red feather pigmentation of many bird species [1] plays pivotal roles in social signaling and mate choice [2, 3]. To produce red pigments, birds ingest yellow carotenoids and endogenously convert them into red ketocarotenoids via an oxidation reaction catalyzed by a previously unknown ketolase [4–6]. We investigated the genetic basis for red coloration in birds using whole-genome sequencing of red siskins (*Spinus cucullata*), common canaries (*Serinus canaria*), and “red factor” canaries, which are the hybrid product of crossing red siskins with common canaries [7]. We identified two genomic regions introgressed from red siskins into red factor canaries that are required for red coloration. One of these regions contains a gene encoding a cytochrome P450 enzyme, *CYP2J19*. Transcriptome analysis demonstrates that *CYP2J19* is significantly upregulated in the skin and liver of red factor canaries, strongly implicating *CYP2J19* as the ketolase that mediates red coloration in birds. Interestingly, a second introgressed region required for red feathers resides within the epidermal differentiation complex, a cluster of genes involved in development of the integument. Lastly, we present evidence that *CYP2J19* is involved in ketocarotenoid formation in the retina. The discovery of the carotenoid ketolase has important implications for understanding sensory function and signaling mediated by carotenoid pigmentation.

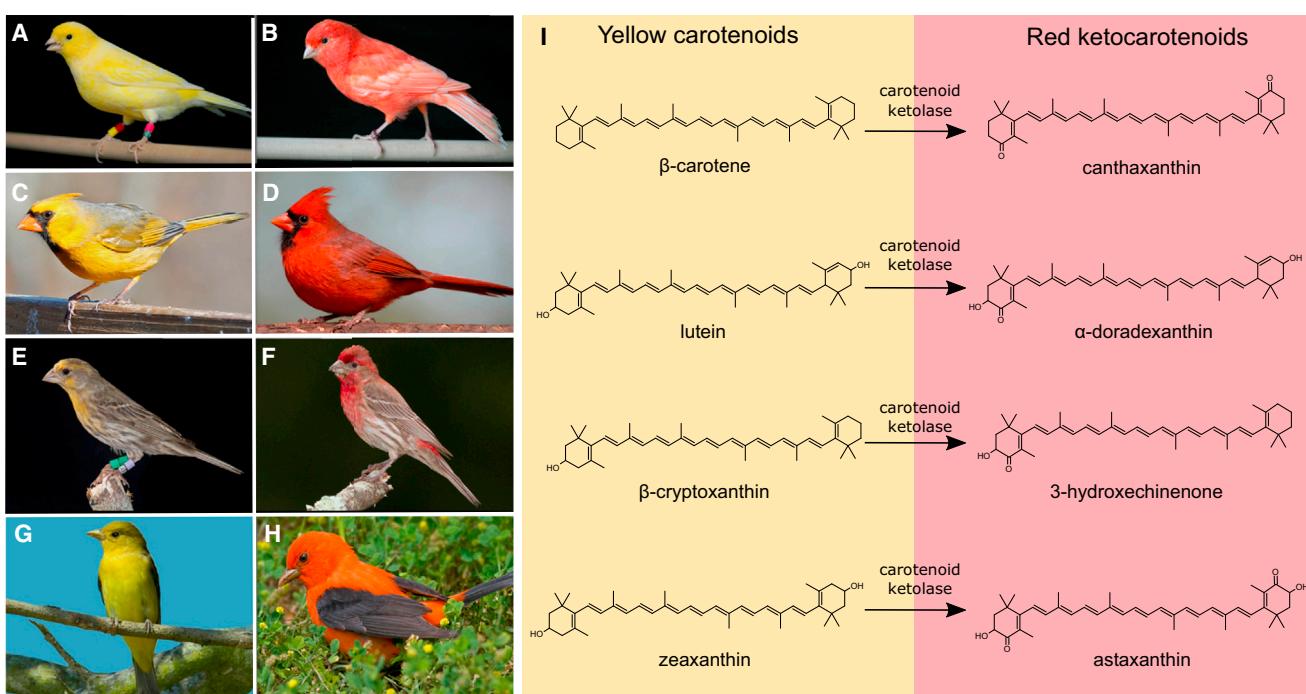
## RESULTS AND DISCUSSION

To identify the genetic basis of red coloration in birds (Figure 1), we took advantage of the unique genetic history of “red factor” canaries. Starting in the 1920s, bird fanciers crossed yellow

common canaries with the red siskin, a South American bird with red ketocarotenoid-pigmented feathers [7]. Hybrid offspring were then backcrossed with common canaries over multiple generations to create the world’s first red factor canary (Figure 2A). Given this genetic history, we reasoned that the genome of red factor canaries would contain regions responsible for red coloration introgressed from red siskins onto a background of common canary DNA. To identify these introgressed regions, we performed whole-genome sequencing of pooled DNA samples from red factor canaries, common canaries (both domestic and wild), and red siskins (Table S1). We generated a total of ~1.5 billion sequence reads that were mapped to the canary reference genome, resulting in an average effective coverage of 19.3× per pool (Table S1).

To detect signatures of genetic differentiation between red factor and common canaries, we measured the fixation index ( $F_{ST}$ ), a metric for summarizing allele frequency differences between populations [12]. We averaged  $F_{ST}$  values across the genome using a sliding-window approach and found that the average level of genetic differentiation was low ( $F_{ST} = 0.079$ ) (Figure 2B), permitting us to detect regions of heightened differentiation indicative of positive selection. The strongest signals of selection in our sliding-window analysis were restricted to two genomic regions (Figure 2B): one located on scaffold NW\_007931131, homologous to zebra finch chromosome 8 (~24,000,000–25,600,000 bp), and the other located on scaffold NW\_007931203, homologous to zebra finch chromosome 25 (~700,000–900,000 bp). All windows above the 99.9<sup>th</sup> percentile of the empirical distribution ( $F_{ST} \geq 0.45$ ) map to these two regions.

Next, we searched for consistent differences in allele frequencies of individual SNPs between two distinct breeds of red factor canaries and five breeds of common canaries. Using a Cochran-Mantel-Haenszel (CMH) test [13], we evaluated 9,414,439 SNPs and found that 15,681 SNPs (0.17%) were significantly associated with red coloration after Bonferroni correction. Importantly, 10,216 of the significant SNPs (65.1%) and all of the top 100 SNPs ( $p \leq 2.43 \times 10^{-18}$ ) localized to the same two genomic regions revealed by the  $F_{ST}$  analysis (Figure 2C).



**Figure 1. Red Feather Coloration Is Mediated by Carotenoid Ketolation**

(A) Yellow common canaries (*Serinus canaria*) lack red ketocarotenoids in their feathers.

(B) Red factor canaries have ketocarotenoid-pigmented plumage. This breed is the product of hybridization between the yellow common canary and the red siskin (*Spinus cucullata*) [7].

(C and D) Rare mutant northern cardinal males (*Cardinalis cardinalis*; C) lack the red ketocarotenoid-containing feathers worn by wild-type males of the species (D) [8].

(E and F) Male house finches (*Haemorhous mexicanus*) have feather coloration ranging from yellow (E) to red (F). Plumage redness is proportional to the abundance of ketocarotenoids [9, 10].

(G) Nonbreeding male scarlet tanagers (*Piranga olivacea*) have yellow plumage.

(H) Breeding males grow red ketocarotenoid-based plumage [5, 11].

(I) Examples of the metabolic conversions used by birds to produce ketocarotenoids from yellow dietary precursors via the action of a carotenoid ketolase.

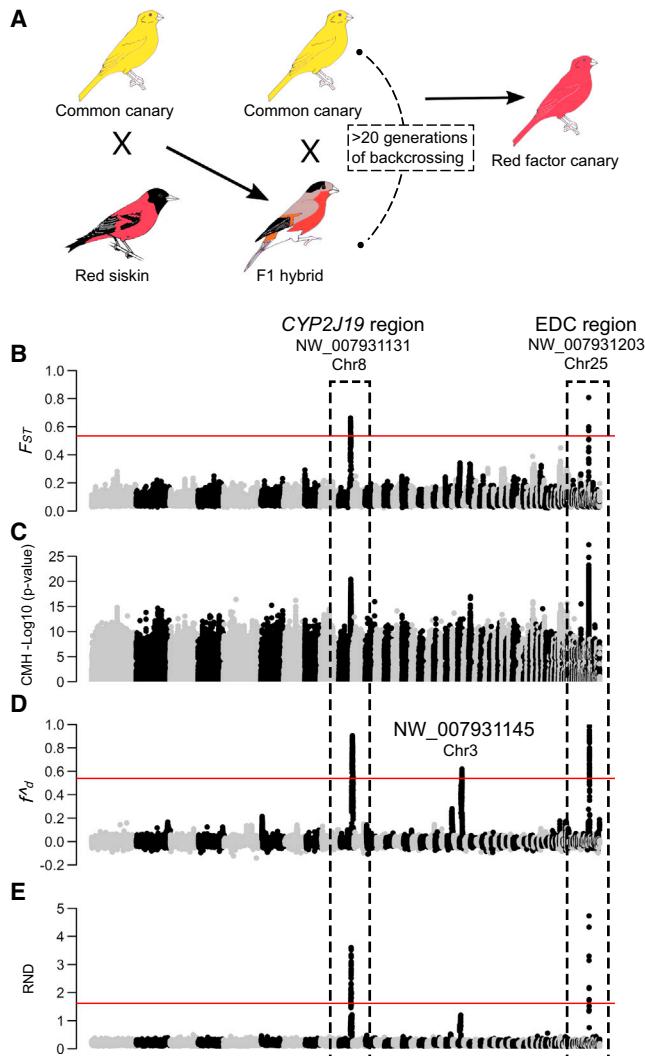
Photo credits: Rebecca J. Koch (A and B), Jim McCormac (C), and Geoffrey E. Hill (D–H).

To locate genomic segments of red siskin origin across the red factor canary genome, we used summary statistics that enabled us to quantify levels of introgression. We started by comparing the genomes of non-red canaries to that of the red siskin. We found that the two species are well differentiated (average nucleotide divergence = 1.77%) and the genomes are well sorted, with 99.4% of all the possible 20 kb windows in the genome displaying at least one diagnostic mutation. This sharp differentiation means that introgressed segments in the red factor genome should be unambiguously identifiable.

We then computed a statistic ( $f^{\wedge}_d$ ) that measures the fraction of the genome shared through introgression [14]. This statistic varies between 0 (no introgression) and 1 (complete replacement). When averaged across the entire genome,  $f^{\wedge}_d$  was close to 0 ( $f^{\wedge}_d = 0.006$ ), suggesting that the overall genetic contribution of red siskin to the red factor canary genome is small, which is consistent with historical records indicating that many generations of backcrossing canary-siskin hybrids to common canaries were necessary to both fix the red trait and improve hybrid fertility [7]. However, the sliding-window analysis identified several segments of the genome with elevated  $f^{\wedge}_d$  values

(Figure 2D), indicative of introgression of red siskin haplotypes in specific genomic regions. The two strongest signals of introgression overlapped the same two top regions in the  $F_{ST}$  analysis and CMH test (NW\_007931131 and NW\_007931203). A third outlier region emerged from this analysis located on scaffold NW\_007931145, which is homologous to zebra finch chromosome 3 (~24,100,000–26,950,000 bp).

The relative node depth statistic (RND) was also calculated between red factor and non-red canaries. RND is a measure of genetic divergence that controls for mutation rate variation, thus allowing us to distinguish between low mutation rate and introgression as the cause of sequence similarity [15]. This analysis pinpointed the same outlier regions, corroborating the previous findings from the  $f^{\wedge}_d$  statistic (Figure 2E). Overall, the substantial overlap between differentiation and introgression statistics indicates that the outlier regions identified here are strong candidates for the genomic regions mediating red coloration in canaries. Furthermore, the fact that at least two genomic regions are implicated in red coloration in canaries (see below) is consistent with the genetic architecture of this trait, which is known to be governed by more than one locus [16].



**Figure 2. The Origin of Red Canaries and Genome-wide Scans for Directional Selection and Introgression**

(A) Red factor canaries were created by crossing common canaries with red siskins. Hybrids were backcrossed with common canaries for many generations to improve the fertility of the line and to remove all siskin characteristics except red coloration. The result is a phenotypically normal canary but with the capacity to produce red ketocarotenoids from yellow dietary carotenoids. (B)  $F_{ST}$  scan across the genome between red and non-red breeds using whole-genome sequencing data (see Table S1). Each dot represents  $F_{ST}$  averaged over 20 Kb windows and iterated in steps of 10 Kb across each scaffold. The 99.9<sup>th</sup> percentile of the empirical distribution is shown as a red horizontal line. (C)  $-\log_{10}$  values of the CMH statistic for every polymorphic SNP through pairwise comparison of allele frequencies between red and non-red canaries. (D)  $f^d$  values summarized in non-overlapping windows of 100 SNPs. The 99.9<sup>th</sup> percentile of the empirical distribution is shown as a red horizontal line. (E) RND values between red and non-red canaries summarized in non-overlapping windows of 10,000 positions (both polymorphic and monomorphic). The 99.9<sup>th</sup> percentile of the empirical distribution is shown as a red horizontal line. The different scaffolds are presented on the x axis in the same order as they appear in the canary reference genome assembly.

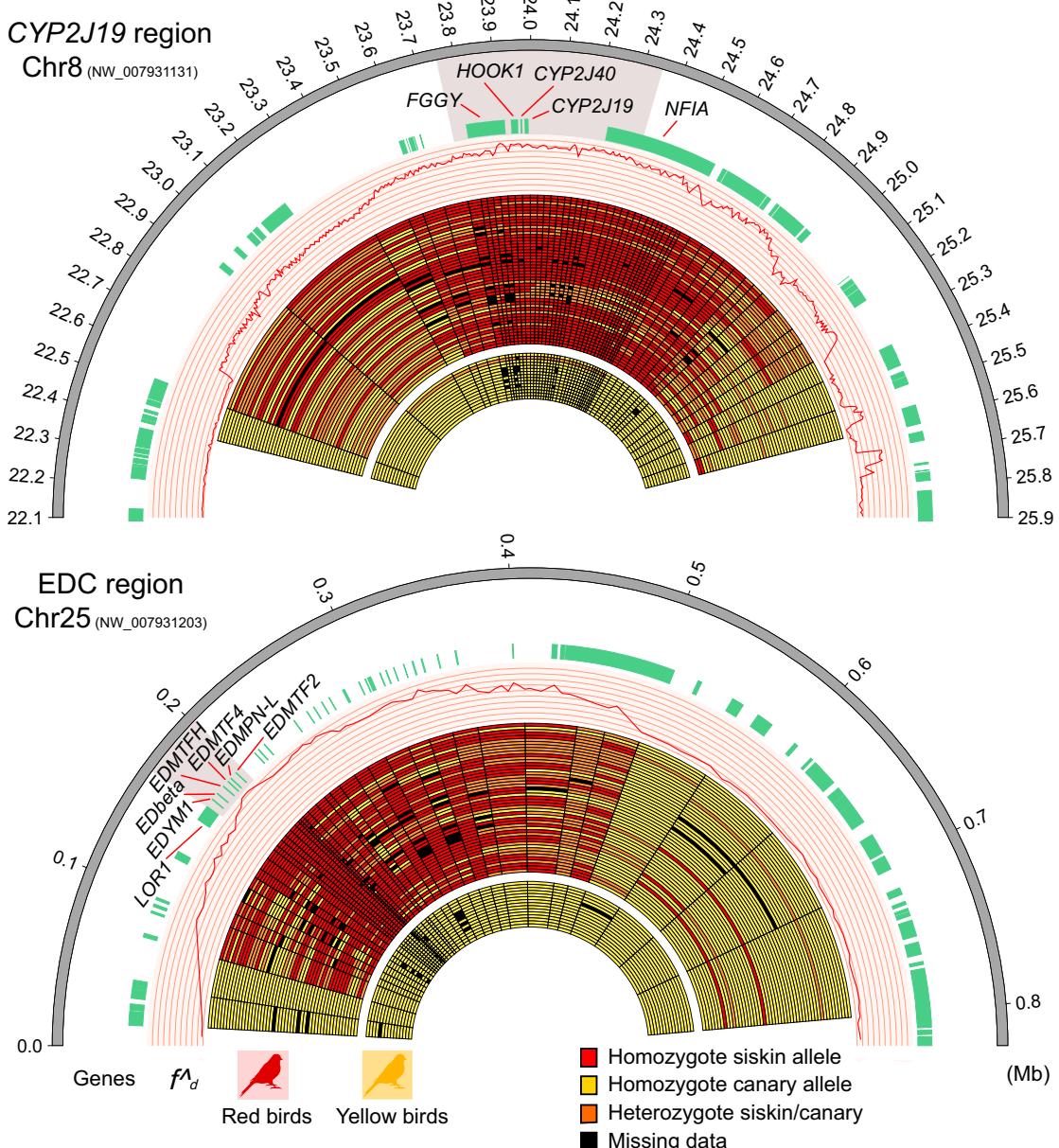
To conduct fine-mapping of these outlier regions, we identified a set of variants that unambiguously distinguish the red siskin and non-red-canary genomes. We then genotyped these vari-

ants in a larger cohort of birds representing a broader range of breeds. For the regions homologous to zebra finch chromosomes 8 (NW\_007931131) and 25 (NW\_007931203), we were able to substantially reduce the size of the candidate intervals (Figure 3). For NW\_007931131, all red birds ( $n = 49$ ) carried at least one haplotype of siskin origin over a 631 kb segment (positions 23,728,812–24,359,539 bp). For NW\_007931203, all red birds were homozygous for the red siskin haplotype encompassing 34 kb (positions 169,696–203,507 bp). In the region homologous to zebra finch chromosome 3 (scaffold NW\_007931145), many red birds did not carry any haplotype of red siskin origin, making it unlikely that any gene in this region is strictly necessary for red coloration (Figure S1). Together with the whole-genome sequencing results (Figure 2), these data indicate that NW\_007931145 contains haplotypes that were introgressed from red siskin but that are segregating at moderate frequencies in red factor canaries (Figure 2B).

Two additional observations from these genotyping results and from animals generated by crossing yellow and red factor canaries provide information about the genetic architecture of red coloration. First, we identified a bird displaying yellow plumage that was heterozygous for the NW\_007931131 region but homozygous for the common canary allele in the NW\_007931203 region. Second, individuals that are heterozygous for both candidate regions do not express red, but rather a yellow/orange coloration. We can thus conclude that both regions are necessary to produce red coloration. The NW\_007931131 region appears to act in a dominant fashion such that a single copy of the red siskin allele is sufficient, whereas homozygosity for the siskin allele in the NW\_007931203 region is necessary to express red feather coloration.

We next investigated the gene content of the two introgressed regions that are necessary for red coloration. Using the published annotation of the canary reference genome, together with an in-depth annotation analysis derived from de novo transcriptome assemblies obtained separately for red and yellow canaries, we identified five genes within the interval on scaffold NW\_007931131: *FGGY*, *HOOK1*, *CYP2J19*, *CYP2J40*, and *NFIA*. Within the interval on scaffold NW\_007931203, we identified six genes—*EDMY1*, *EDbeta*, *EDMTFH*, *EDMTF4*, *EDMPN-L*, and *EDMTF2*—plus the promoter region of one gene (*LOR1*) (detailed in Table S2). The latter interval maps to the epidermal differentiation complex (EDC), an extended block of genes involved in integumentary development [18]. We also utilized whole-genome sequencing data to detect structural variants (i.e., deletions, duplications, inversions, or translocations) that differ between the common canary and red siskin haplotypes (Table S3). We identified several candidate variants, but none overlapped annotated genes, suggesting that the gene content and synteny of both regions is likely to be preserved between the two divergent haplotypes. However, without high-quality genome assemblies for both species, we cannot absolutely exclude the existence of some minor structural differences.

The skin and liver are the two most important anatomical sites for conversion of yellow dietary carotenoids into red ketocarotenoids for plumage coloration in birds [19]. Thus, to further pinpoint candidate causal genes in the introgressed

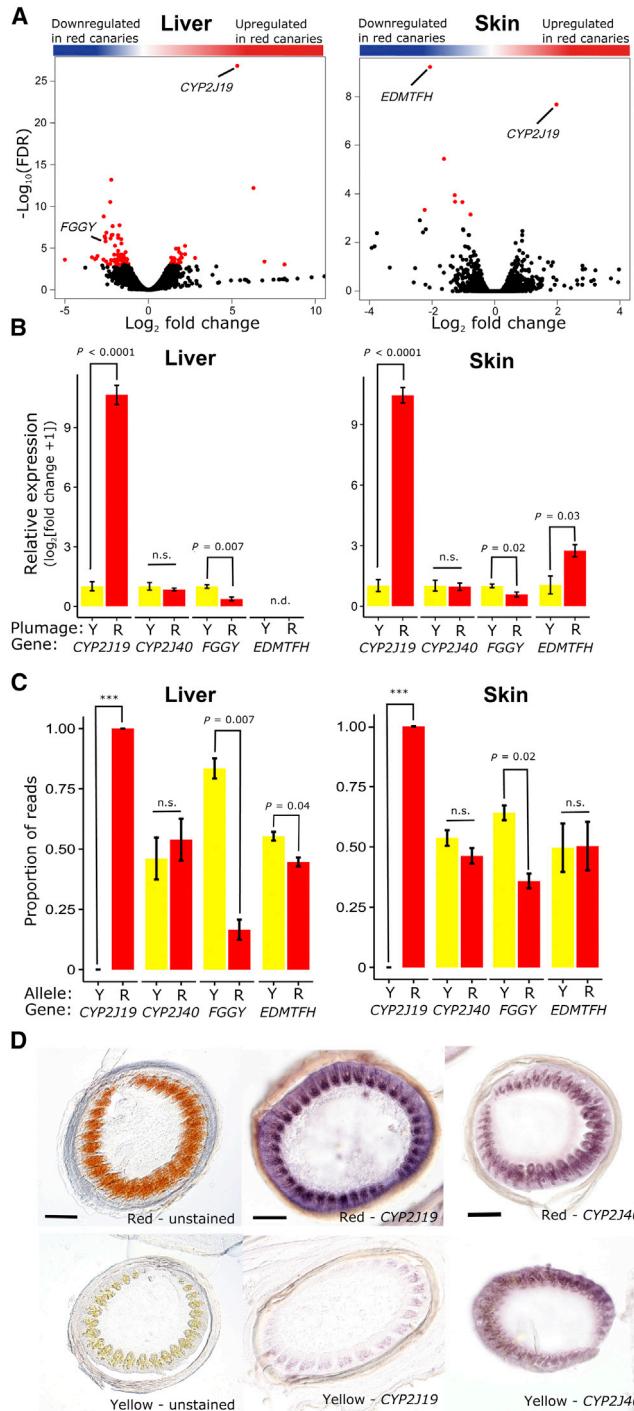


**Figure 3. Fine Mapping of Introgressed Segments from Red Siskin Origin in the Red Canary Genome**

The outermost semi-circle represents the genomic coordinate in megabases. The next semi-circle (from the outside inward) shows the location of genes from the canary genome annotation (green boxes). In the next semi-circle,  $f^d$  values are shown (solid red line). The innermost semi-circles represent the genotyping results for SNPs found to be diagnostic between red siskin and common canaries in 49 red canaries and 15 non-red canaries, as indicated by red and yellow canary silhouettes, respectively. Each row represents one individual, and individuals appear in the same order on both Chr8 and Chr25 graphs. Red, yellow, and orange squares indicate positions homozygous for the red siskin allele, homozygous for the yellow canary allele, and heterozygous for both alleles, respectively. Missing data are represented by black boxes. Light-gray highlighting indicates the longest continuous regions where all red individuals carry at least one copy of the red siskin haplotype. Only the names of genes within these regions are shown (see Table S2). The circular plot was generated using Circos [17]. See also Figure S1.

regions, we analyzed differential gene expression between red and yellow canaries in both adult skin (plucked 10 days prior to induce feather regeneration) and liver by RNA sequencing (RNA-seq; Table S4). We observed nine genes in the skin and 102 genes in the liver that were differentially expressed (false discovery rate [FDR] = 0.1%) (Figure 4A). Within the two candi-

date regions associated with red coloration, we detected significant differential expression of three genes in liver and/or skin: *CYP2J19*, *FGGY*, and *EDMTFH* (Figure 4A). To corroborate the RNA-seq results, we conducted qPCR. We found *CYP2J19* to be expressed at more than 1,000-fold higher levels in both skin and liver of red canaries compared to yellow canaries,



**Figure 4. Levels and Patterns of Gene Expression in Red and Yellow Canaries**

(A) Volcano plot of statistical significance after FDR correction (y axis) against log<sub>2</sub> fold change in expression (x axis) for both liver and skin using RNA-seq (see Table S4). Significant genes are depicted as red dots, and those that also overlap the candidate regions are labeled.

(B) qPCR analysis of the transcript levels of four genes (CYP2J19, CYP2J40, FGGY, and EDMTFH) measured in the liver and skin of three red (R) and three yellow (Y) canaries. Expression is presented as the mean ± SD of fold change relative to the mean expression level in the respective tissue of the yellow canaries.

whereas FGGY had 2- to 3-fold lower expression in red birds compared to yellow (Figure 4B). In contrast, we found no detectable expression of EDMTFH in liver and slightly higher levels in the skin of red birds compared to yellow birds (Figure 4B). This latter result conflicts with the results from RNA-seq, which showed EDMTFH to be downregulated in red skin. Upon reexamination of the RNA-seq data, we discovered that the apparent differential expression observed for EDMTFH was due to a read-mapping artifact, in which red-siskin-derived EDMTFH reads mapped less efficiently to the common canary transcriptome assembly. Thus, we conclude that CYP2J19 expression is markedly elevated in the skin and liver of red birds relative to yellow birds, but that FGGY and EDMTFH levels are only moderately different between the two.

To further characterize these expression differences, we crossed red factor canaries to common canaries and measured allele-specific expression in the offspring. Strikingly, we found that 100% of CYP2J19 transcripts were derived from the red allele in both skin and liver in all three offspring examined (Figure 4C). Additionally, we found preferential expression of the yellow allele of FGGY in both skin (~83% of normalized reads deriving from the yellow allele) and liver (~64% of normalized reads deriving from the yellow allele). In contrast, we found similar levels of expression of yellow and red alleles of EDMTFH in skin and liver (although EDMTFH was undetectable in liver by qPCR, it was possible to amplify trace amounts of the transcript by PCR for allelic analysis). Thus, the most notable finding from these studies was the occurrence of extreme allelic imbalance favoring expression of the red-siskin-derived allele of CYP2J19 in both skin and liver.

Unlike EDMTFH, which encodes a structural protein with no known enzymatic function [18], both FGGY and CYP2J19 are predicted to encode enzymes and therefore have the potential to mediate the formation of red ketocarotenoids from yellow precursors. Because skin and liver are the two most likely sites of feather ketocarotenoid production in birds [19], a carotenoid ketolase should be upregulated in red bird skin and/or liver relative to yellow birds. Expression of FGGY is significantly lower in the red factor canary than in the yellow canary, the opposite of what one would expect for a ketolase. Furthermore, FGGY encodes a protein with homology to a family of kinases that phosphorylate carbohydrate substrates [20], a function seemingly

(C) Quantitation by allele-specific RNA-seq of four genes (CYP2J19, CYP2J40, FGGY, and EDMTFH) measured in the liver and skin of three F1 red × yellow hybrid canaries. Allele expression is presented as the mean ± SD of the proportion of red and yellow allele read counts for each gene. Significance was determined with a one-sample Student's t test with the null hypothesis of  $\mu = 0.5$ . \*\*\* indicates that 100% of the reads were from the red allele.

(D) Unstained sections and *in situ* hybridization of regenerating feather follicles (10 days post-pluck) of red and yellow canaries. Reddish-orange ketocarotenoid pigmentation is evident in the developing barb ridges of red canary feather follicles, whereas fainter yellow carotenoid-based pigmentation is evident in the yellow feather (leftmost panels). *In situ* hybridization probes for CYP2J19 and CYP2J40 also localize to the barb ridge of the developing feather. CYP2J19 expression is markedly elevated in the red canary feather follicles compared to the yellow (middle panels), whereas comparable levels of CYP2J40 expression are seen in red and yellow feathers (rightmost panels). Scale bars, 100  $\mu$ m.

See also Figures S2 and S3.

unrelated to the lipid oxidation required of a ketolase. Although we cannot exclude a role for FGGY in the production of red feathers, these considerations indicate that it is not an ideal candidate for the ketolase.

*CYP2J19*, in contrast, is markedly elevated in the red factor canary and is a member of a large superfamily of cytochrome P450 oxygenases that act on a range of small-molecule substrates, including carotenoids [21–23]. Members of this family are known to mediate ketolation in the 4 position of the β-ionone ring of retinoids in mammals [24]. Furthermore, a cytochrome P450 family member, crtR, is required for astaxanthin production from β-carotene in the yeast *Xanthophyllomyces dendrorhous* [25].

If skin is a site of ketocarotenoid production in red canaries and *CYP2J19* encodes the carotenoid ketolase, then we expect higher level of *CYP2J19* expression in the regenerating feather follicles of red birds compared to yellow birds. To investigate this possibility, we analyzed the expression of *CYP2J19* in skin by *in situ* hybridization. We found strong expression of *CYP2J19* in the regenerating feather follicle of red factor canaries, whereas transcripts were present at much lower levels in the skin of yellow birds (Figure 4D). The pattern of *CYP2J19* expression correlates precisely with the distribution of ketocarotenoids in the regenerating feather (Figure 4D). For comparison, we also analyzed the expression of *CYP2J40* and found comparable levels in the regenerating feather follicles of red and yellow birds (Figure 4D). Overall, these findings make *CYP2J19* a strong candidate for the avian carotenoid ketolase.

Because ketocarotenoids are also present in the avian retina, we investigated whether *CYP2J19* might be expressed in this tissue in a pattern consistent with a role in carotenoid ketolation. The red single cone in the avian retina utilizes a ketocarotenoid, astaxanthin, as an intracellular spectral filter to enhance color discrimination [26–28]. Astaxanthin is thought to be synthesized locally within the red cone from a yellow dietary precursor, zeaxanthin (Figures 1 and S2A). We found that *CYP2J19* transcript levels in the chicken retina correlate well with the levels of astaxanthin over development (Figures S2B, S2D, and S2E). Furthermore, *in situ* hybridization indicates that *CYP2J19* expression is restricted to a subset of cells in the outer nuclear layer, consistent with expression in red single cones (Figure S2C). This correlation is compatible with the hypothesis that *CYP2J19* mediates production of astaxanthin in the developing red cone.

Interestingly, yellow canaries have levels of *CYP2J19* expression in the retina similar to those found in the retinas of red birds (Figure S3). This finding suggests that the expression differences observed between yellow and red birds in the skin and liver are due to *cis*-regulatory differences between the two *CYP2J19* alleles. The vast majority of bird species possess ketocarotenoid-based oil droplets in their retinas and thus presumably express *CYP2J19* in this tissue. However, only a subset of bird species deposit these red pigments in their skin and feathers. Thus, we hypothesize that nearly all bird species have the latent capacity to produce ketocarotenoid-based red feather coloration, but that *cis*-regulatory changes at the *CYP2J19* locus are required for the gain of expression in the skin and/or liver that leads to the emergence of this trait in selected species.

Homozygosity for red siskin alleles in the EDC region is also necessary for red feather production in canaries. If *CYP2J19* encodes the ketolase, then what is the function of the EDC region? We did not identify major differences in gene expression within the EDC region between red and yellow birds, suggesting that coding variants might be involved. To address this point, we scanned all six candidate genes in the EDC region for coding variants. We identified 11 nonsynonymous variants in three genes (*EDMTFH* [12], *EDMPN-L* [1], and *EDMTF2* [2]) (Table S5). In addition, we evaluated the five candidate genes in the *CYP2J19* region for coding variants and found another 24 nonsynonymous mutations in three genes (*HOOK1* [7], *CYP2J40* [7], and *CYP2J19* [14]). We did not identify frameshift or STOP loss/gain mutations in any of the genes. Based on sequence conservation, we predict that several amino acid changes for which the derived state is present in red canaries might alter protein function (Table S5), but functional assays will be required to determine what role, if any, these coding variants play in red feather coloration.

The enzyme that converts yellow carotenoids into red ketocarotenoids in birds has long been sought [5, 7, 29, 30]. Here, we present *CYP2J19* as a strong candidate for the carotenoid ketolase. Genetic and expression analysis in canary and chicken support *CYP2J19* as the ketolase, or a component thereof. In a co-submitted paper, *CYP2J19* was also identified as the gene responsible for red coloration in the bill and legs of zebra finches [31], suggesting that it serves as the ketolase in multiple tissues across diverse groups of birds. Carotenoid coloration is widely accepted as a condition-dependent signal of individual quality that is assessed in mate choice and other social interactions [32, 33]. The discovery of the ketolase gene in birds presents unprecedented opportunities for investigating the signal content of carotenoid coloration [34]. The oxidation potential required by P450 enzymes suggests that production of red pigments via *CYP2J19* will be sensitive to the organism-wide oxidative state, potentially explaining why red carotenoid coloration is so consistently linked to individual quality [35, 36].

## ACCESSION NUMBERS

The accession number for the whole-genome resequencing and RNA-seq read data reported in this paper is GenBank: SRP065487. The accession number for the SNP genotyping data and de novo transcriptome assemblies reported in this paper is Dryad: <http://dx.doi.org/10.5061/dryad.sm12c>.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2016.03.076>.

## AUTHOR CONTRIBUTIONS

M.C., J.C.C., and G.E.H. conceived and coordinated the study with input from R.J.L. and L.A. M.C., J.D.J., M.S.F., and J.M.-F. conducted the bioinformatic analyses of the whole-genome resequencing and RNA-seq datasets. M.C., R.J.L., and P.M.A. maintained the canaries and performed the controlled crosses. M.B.T. and J.C.C. designed and performed qPCR, *in situ* hybridization, allele-specific expression experiments, and retinal analyses. M.C., J.C.C., and G.E.H. wrote the paper with input from other authors.

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