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The Dark brown plumage color in chickens is caused by an 8.3-kb deletion upstream of *SOX10*

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Summary

The *Dark brown (DB)* mutation in chickens reduces expression of black eumelanin and enhances expression of red pheomelanin, but only in certain parts of the plumage. Here, we present genetic evidence that an 8.3-kb deletion upstream of the *SOX10* transcription start site is the causal mutation underlying the DB phenotype. The *SOX10* transcription factor has a well-established role in melanocyte biology and is essential for melanocyte migration and survival. Previous studies have demonstrated that the mouse homolog of a highly conserved element within the deleted region is a *SOX10* enhancer. The mechanism of action of this mutation remains to be established, but one possible scenario is that the deletion leads to reduced *SOX10* expression which in turn down-regulates expression of key enzymes in pigment synthesis such as tyrosinase. Lower tyrosinase activity leads to a shift toward a more pheomelanistic (reddish) plumage color, which is the characteristic feature of the DB phenotype.

Introduction

In natural populations of birds, there is an extensive diversity of plumage color among species in contrast to remarkably uniform plumage color within species. We have good reasons to believe that a large proportion of the diversity between species is because of regulatory changes affecting the expression of genes involved in pigment synthesis. The reason for this assumption is that it is the pattern of pigmentation that varies between species rather than the ability to produce pigment. Biosynthesis of the two types of melanin

(brown/black eumelanin and yellow/red pheomelanin) takes place in melanosomes (Kushimoto et al., 2003; Le Douarin and Dupin, 2003; Wakamatsu and Ito, 2002). Genetic changes that alter plumage color can affect several different processes, such as the migration and survival of melanocytes, melanosome function and transport, and pigment synthesis.

Melanocytes in the skin are neural crest-derived cells, and recent studies have revealed that they develop through two alternative routes, either directly from neural crest cells or via an indirect route from Schwann cell precursors (Ernfors, 2010). The transcription factors

Significance

SOX10 is an important transcription factor in melanocytes and some other cell types. *SOX10* loss-of-function mutations show pigmentation defects in the heterozygous condition and lethality in homozygotes. Here, we show that an 8.3-kb deletion located 14 kb upstream of *SOX10* is causing the fully viable Dark brown plumage color in chickens. The *Dark brown* allele is particularly interesting because it affects the pattern of pigmentation rather than the presence/absence of pigmentation. The access to a simple diagnostic test for determining the *DB* genotype will facilitate the dissection of other loci contributing to the fascinating feather pattern variation observed in chickens.

SOX10 (SRV-box 10) and microphthalmia-associated transcription factor (MITF) have crucial roles during the development of melanocytes, and mutations in these genes lead to pigmentation defects in both birds and mammals (Harris et al., 2010; Levy et al., 2006; Mochii et al., 1998).

The *Dark brown* (*DB*) locus was originally defined in autosomally barred Fayoumi chickens (Moore and Smyth, 1972). Two alleles were defined at this locus, *wild-type* (*DB*^N*) and *Dark brown* (*DB*^{DB}*). Birds with the *DB* phenotype have a pattern similar to the black-tailed red phenotype and with a columbian-like restriction of the expression of eumelanin (Smyth, 1996). In males, the breasts are brown/red, and in females, the pheomelanin appears as an orange-tan or burnt orange color. *DB* was previously mapped to chicken chromosome 1 (Bitgood and Somes, 1990). Earlier studies have indicated that the expression of the *DB* allele is modified by alleles at the *Extension* (*E*) locus in chicken (Bitgood and Somes, 1990; Campo and Alvarez, 1988; Moore and Smyth, 1972; Moore et al., 1978). *E* encodes the melanocortin 1-receptor (MC1R) which regulates the relative proportion of eumelanin and pheomelanin (Kerje et al., 2003).

In this study, we present conclusive evidence that the *DB* phenotype in chicken is caused by an 8.3-kb deletion located 14 kb upstream of *SOX10*.

Results

Phenotyping of the *DB* plumage color

We have used an intercross between the red junglefowl (RJF) and the obese strain (OS) of chicken to dissect the inheritance of plumage color. A number of plumage color phenotypes were observed among the 756 individuals in the F₂ generation of this cross. Segregation was observed at the *Extension* (*E*) locus (Kerje et al., 2003), the *Dominant white* (*I*) locus (Kerje et al., 2004), and the *Sex-linked barring* (*B*) locus (Hellström et al., 2010). In addition to these phenotypes, an orange/red phenotype stood out. This phenotype was in good agreement with the previously described phenotypic effect of the *DB* allele. The *DB* phenotype was strictly scored by examining pictures for each bird at different ages, 161 of 756 individuals were scored as *DB*. Males having a bright brown/red/orange breast and a non-pheomelanistic tail were scored as *DB* (Figure 1A). Two categories of the *DB* phenotype were scored in males, one with a more extreme phenotype (type 1, 62 individuals) and one less apparent phenotype (type 2, 58 individuals). The 41 (out of 369) females scored as *DB* had an orange-tan color all over the body except for the tail feathers.

Assignment of *DB* to the *SOX10* region

Our initial linkage analysis was carried out with the assumption that *DB* was inherited as a recessive trait. A

combined analysis with data from both males and females gave highly significant logarithm of odds (LOD) scores for an assignment to chromosome 1 in line with the previously reported linkage data for *DB* (Bitgood and Somes, 1990). However, a closer examination of the data showed that there was complete linkage to this region in females, but indicated a considerable amount of recombination in males. A CHROMPIC analysis using the CRIMAP software indicated that these male 'recombinants' were rather phenotyping errors in males or an incorrect genetic model for the inheritance of *DB*. We therefore restricted our initial linkage analysis to females.

The 41 *DB* females were scored as homozygous *DB/DB*. The remaining animals were scored as genotype unknown, because we could not determine whether a bird did not express the *DB* phenotype because of the action of the *wild-type* allele or because alleles at other loci masked the expression of *DB*. Pairwise linkage analysis of *DB* against 356 single nucleotide polymorphism (SNP) markers evenly spread across the genome resulted in highly significant LOD scores for markers on chromosome 1 (Table 1). The interval harboring *DB* was defined based on the closest flanking markers showing recombination and extended from marker rs13651949 at 50.4 Mb to marker rs14827727 at 57.1 Mb on chromosome 1 (<http://www.genome.ucsc.edu>, chicken May 2006 assembly), a region containing 56 RefSeq genes. No recombination and a LOD score of 14.5 were obtained for marker rs13866016 at 53.4 Mb located in the middle of the interval (Table 1). In the defined interval, *SOX10* with a transcription start site at 53.0 Mb was identified as a strong positional candidate gene.

All *SOX10* exons were sequenced from *DB/DB* and *wild-type* homozygotes. We identified several informative SNPs, but none was exclusively associated with the phenotype. We then decided to resequence evolutionary conserved regions flanking *SOX10*. This resulted in the discovery that the region corresponding to the region deleted in *Hry* mice (Antonellis et al., 2006) did not amplify in individuals with the *DB* phenotype. Further analysis revealed a deletion of 8268 bp and an accompanying insertion of 10 bp, referred to in the text as an 8.3-kb deletion, associated with the *DB* allele that deleted one highly conserved non-coding sequence (Figure 1B). In addition to the conserved element, an exon of a Genscan predicted gene was indicated within the deleted area (Figure 1B). Primers were designed to try to amplify this sequence, but RT-PCR analysis using cDNA from growing feathers did not give any amplification product from any genotype (data not shown). There is no corresponding gene in this region in other species, and the predicted gene is not supported by any spliced mRNA sequence from chicken, so it is likely that this is a false gene prediction.

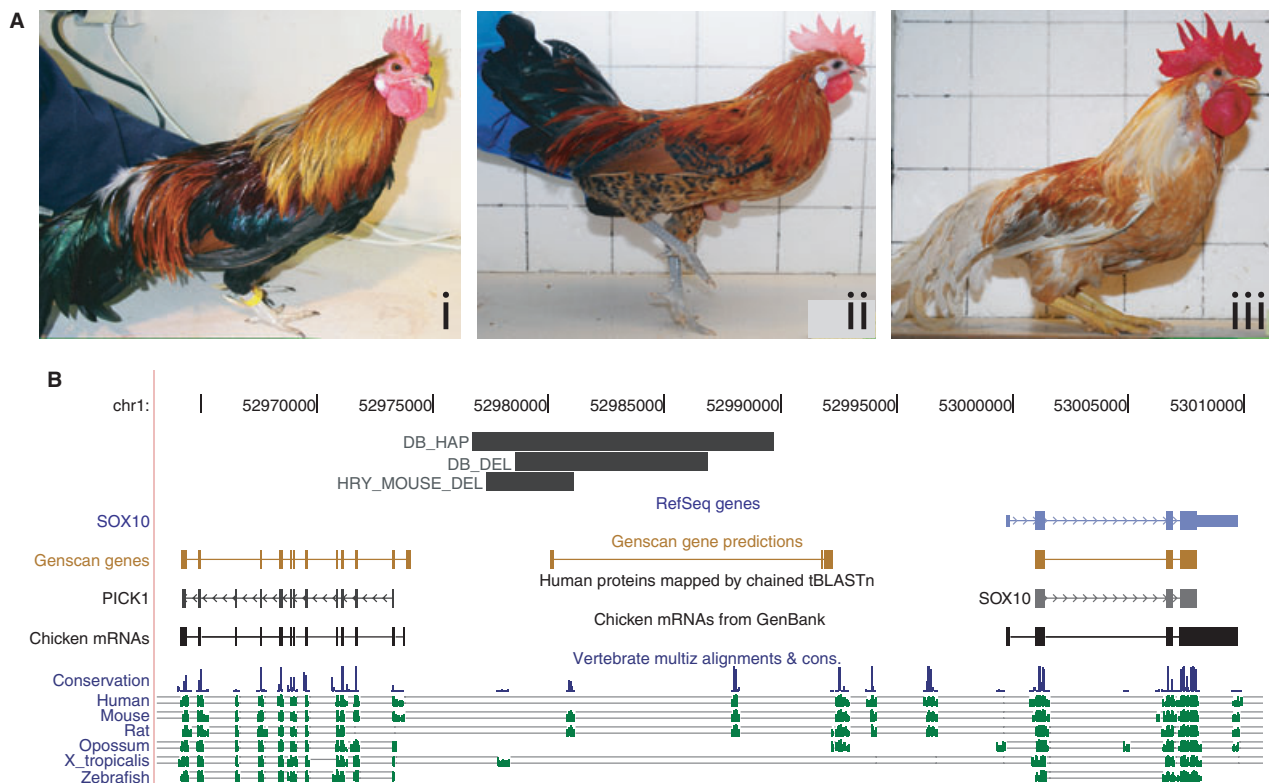


Figure 1. (A) Illustration of the Dark brown (DB) phenotype. (i) A red junglefowl male representing the wild-type phenotype having a black breast, (ii) a DB male showing a red/orange breast and autosomal barring, (iii) a DB male showing a red/orange breast on the *Dominant white* background, which inhibits the expression of eumelanin. (B) Localization of the 12.8-kb haplotype showing complete association with the *DB* allele across populations (DB_HAP). The 8.3-kb deletion (DB_DEL) upstream of *SOX10* is also indicated. Furthermore, the region homologous to the deleted region in the *Hry* mouse (HRY_MOUSE_DEL) including the MCS7 element is marked. The figure also shows the coding parts of *SOX10* and *PICK1*, Genscan gene predictions as well as the degree of sequence conservation among vertebrates. The figure was generated using the chicken genome assembly as presented on the UCSC Genome Browser (<http://www.genome.ucsc.edu>, chicken May 2006 assembly).

Table 1. Assignment of the *Dark brown* (DB) locus to chicken chromosome 1 by two-point linkage analysis in females

Marker	Position (bp)	Rec. fraction	LOD score
rs14815974	43 407 393	0.21	4.0
rs13651949	50 352 140	0.07	13.5
DB_DEL	52 978 565–52 986 833	0	24.7
SOX10_SNPs	53 001 275–53 001 293 ^a	0	19.0
rs13866016	53 352 637	0	14.5
rs14827727	57 124 368	0.13	7.0

^aThree single nucleotide polymorphisms (SNPs) at positions 53 001 275, 53 001 278, and 53 001 293 bp.

The *DB* deletion (DB_DEL) and an informative SNP in exon 2 of *SOX10* (SOX10_SNP) were scored in the entire intercross and used for linkage analysis. Neither DB_DEL nor SOX10_SNP showed any recombination with the *DB* locus (Table 1); DB_DEL gave the highest LOD score, 24.8 because it was 100% informative in this cross.

The genotype distribution for the *DB* deletion for both males and females with the DB phenotype is given in Table 2. Two types of DB males were recognized, type 1 with a more conspicuous reddish plumage than the type 2 males. The data show that DB is inherited as a recessive trait in females because all 41 DB females were homozygous for the *DB* deletion. In contrast, DB showed a codominant inheritance in males. Most type 1 males were homozygous for the deletion, whereas most males with the less conspicuous type 2 phenotype were heterozygous. This explains why the linkage analysis using males gave a number of false recombinants when treating DB as a recessive trait. Six males scored as DB_type 2 were homozygous for the non-deleted *wild-type* allele at the *DB* locus. We are convinced that these represent phenotype misclassifications possibly because of segregation at other loci affecting plumage color combined with the fact that the type 2 phenotype compared with the type 1 class are more difficult to distinguish from the wild type. This was also confirmed by CHROMPIC analysis, which strongly indicated that these six exceptions represented

Table 2. Genotype distribution of the 8.3-kb deletion upstream of *SOX10* (*DB_DEL*) among birds with the Dark brown (DB) phenotype in the F₂ generation of the OSxRJF intercross

Phenotype	Genotype ^a				Total
	<i>wt/wt</i>	<i>wt/DEL</i>	<i>DEL/DEL</i>	No-gt ^c	
DB-male-type 1 ^b	0	13	49	0	62
DB-male-type 2 ^b	6	49	3	0	58
DB-female	0	0	41	0	41
Total, all birds in the intercross	161	322	181	92	756

^aThe OS and red junglefowl parental birds are fixed for different alleles, *DEL*, and *wt* (*wild-type*), respectively.

^bTwo categories of the DB phenotype were scored in males, one with a more intense phenotype (type 1) and one less obvious phenotype (type 2).

^cNo-gt, missing genotype.

phenotyping errors rather than recombination events between *DB* and the deletion.

Identical-by-descent mapping and complete association across populations

The region around the deletion was resequenced in eight animals, two RJFs and six birds from four different breeds of chicken with the DB phenotype (Friesian Fowl, Fayoumi, Westfälischer Totleger and White Leghorn-OS line). This resequencing resulted in the identification of a 12 785-bp-long *DB* haplotype from the flanking markers at position 52 976 756 bp to position 52 989 541 bp. All DB individuals sequenced were homozygous and identical-by-descent (IBD) within this interval, and the minimum shared *DB* haplotype showing homozygosity breaks up at the flanking markers (Table S1). The IBD region contains over 40 polymorphic sites including the 8.3-kb deletion in comparison between *DB* and *wild-type* alleles. Surprisingly, one of the founder RJF males (RJF_923) from the OS/RJF intercross was heterozygous for a haplotype that was identical to the *DB*-associated haplotype except for the deletion and the accompanying 10-bp insertion replacing the deleted sequence (Table S1). RJF_923 is apparently carrying the ancestral haplotype on which the deletion occurred. Because this RJF male is grandsire to about 400 F₂ progeny, we can be absolutely sure that this haplotype does not carry the causal *DB* mutation. We can thus exclude all polymorphisms associated with the *DB* haplotype as being causative except the 8.3-kb deletion and its accompanying 10-bp insertion. Because the IBD haplotype shared between *DB* chromosomes is broken outside the 12 785-bp region, all sequence polymorphisms outside this area including all *SOX10* coding exons can also be excluded. Thus, these results provide conclusive genetic evidence that the 8.3-kb deletion is the causative mutation for the DB phenotype.

The PCR-based diagnostic test for the deletion (*DB_DEL*) was used to screen 14 chicken populations

Table 3. Genotype distribution of the 8.3-kb deletion upstream of *SOX10* causing the Dark brown (DB) phenotype in different populations sorted by phenotype

Breed	<i>wt/wt</i>	<i>wt/DEL</i>	<i>DEL/DEL</i>	Total
DB				
Friesian Fowl	0	0	13	13
Fayoumi	0	0	7	7
White Leghorn (OS line)	0	0	9	9
Westfälischer Totleger	0	0	12	12
Wild-type				
Houdan	5	0	0	5
Dorking	4	0	0	4
Finnish Landrace	4	0	0	4
Red Villafranguina ^a	34	0	0	34
Czech Golden Pencilled	5	0	0	5
Transylvanian Naked Neck	5	0	0	5
Australorp	5	0	0	5
Yurlov	5	0	0	5
Red junglefowl	5	0	0	5
INRA experimental flock	12	0	0	12
Total	84	0	41	125

^aDifferent types of Villafranguina chickens differ in plumage color, but the ones used in this screen did not show the DB phenotype.

with and without the *DB* allele present (Table 3). The deletion was detected in all 41 DB chickens and in none of the 84 tested birds carrying the *wild-type* allele at this locus.

Discussion

This study is an advance in plumage color genetics in chicken because *DB* is one of the major loci controlling plumage color, and it affects the expression of pigment patterns in chicken, such as autosomal barring and spangling (Smyth, 1996). The access to a simple diagnostic test for determining the *DB* genotype will facilitate the dissection of other loci contributing to the fascinating feather patterns observed in chicken.

We show that an 8.3-kb deletion containing an evolutionarily conserved non-coding sequence located 14 kb upstream of the *SOX10* transcription start site is causing the DB phenotype in chicken. This conclusion is supported by mapping data, the complete concordance between the presence of the deletion and the phenotype across breeds, and the fact that the 8.3-kb deletion and the accompanying 10-bp insertion is the only unique sequence difference between *DB* and *wild-type* chromosomes. We propose that the deletion is a *cis*-acting regulatory mutation that affects the expression of *SOX10* because (i) the deleted region does not appear to contain any coding sequence and (ii) it has already been established using transgenic zebrafish experiments that the corresponding sequence in mice constitutes a *SOX10* enhancer (see below). At present, we cannot exclude the possibility that the deletion affects the expression of other genes in the interval, e.g. the

closely linked *PICK1* gene, but none of these has a well-established role in melanocyte biology, whereas *SOX10* has a key role for melanocyte development (Harris et al., 2010). At present, we cannot formally exclude the possibility that the 10-bp insertion creates a regulatory element, but it appears more likely that the 8.3-kb deletion is causal because it contains a highly conserved element corresponding to an established enhancer element in mice.

Compared with coding mutations, it is much more challenging to identify and prove the functional significance of mutations in non-coding regions. This study therefore demonstrates the strength of animal genetics, as we were able to generate conclusive genetic evidence that the 8.3-kb deletion must be the causative mutation. This was achieved because we identified the ancestral haplotype that only differed from the mutant haplotype by the 8.3-kb deletion and the accompanying 10-bp insertion. The possibility to find the ancestral haplotype for a mutation is not negligible in domestic animals because of their relatively young history and the strong phenotypic selection. Thus, the ancestral haplotype may still be segregating in some populations as illustrated in this study. Other spectacular examples where the identification of the causative mutation was greatly facilitated by access to the ancestral haplotype include a single nucleotide substitution in *IGF2* affecting muscle growth in pigs (Van Laere et al., 2003) and a non-coding mutation underlying the callipyge muscle hypertrophy phenotype in sheep (Freking et al., 2002).

The *DB* locus was first described in the 1970s. Adult females were reported to have a distinct orange-tan or burnt orange color, and some males had completely red-brown breasts (Moore and Smyth, 1972; Moore et al., 1978). This phenotypic description fits well with the phenotypes observed in our OSxRJF intercross. In accordance with Campo and Alvarez (1988), our data indicated that the penetrance of the *DB* mutation differed between sexes, we observed a co-dominant inheritance in males and a recessive inheritance in females. All 41 females scored as *DB* were homozygous for the *SOX10* deletion. The main difference between the two classes of *DB* males is that the type 1 group shows a more conspicuous change in plumage color compared with the wild-type. Most type 1 individuals were homozygous for the deletion (49 out of 62), and the remaining were heterozygous for the *DB* allele. Among the males classified as type 2, the distribution was inverted, 49 individuals were heterozygous for the deletion, and only three were homozygous. This difference between genders is not surprising given the strong sexual dimorphism in plumage color in the wild ancestor of the domestic chicken, the red jungle fowl. Similarly, the phenotypic expression of the *Extended black* allele at the *Extension/MC1R* locus showed a major difference between genders in our previous analysis of a RJF/White Leghorn intercross (Kerje et al., 2003).

In 1990, Bitgood and Somes (1990) published linkage data for chicken chromosome 1 where *DB* was mapped between *P* (*Pea-comb*) and *Pg* (*Pencilling*), *P-33-DB-20-Pg*. Wright et al. (2009) confirmed a localization to chromosome 1 when they demonstrated that *Pea-comb* is caused by a mutation in intron 1 of *SOX5*, located around position 68 Mb on this chromosome. In the present study, we show that *DB* is caused by an 8.3-kb deletion located around position 53 Mb.

Antonellis et al. (2006) described a transgenic mouse strain characterized by decreased embryonic *Sox10* expression and a reduction of melanocytes and distal enteric neurons implying a defect in the migration of neural crest-derived cells. These mice (*Sox10^{Hry}*) were found to have a 15.9-kb deletion of an evolutionary conserved non-coding sequence 47 kb upstream of the *Sox10* transcription start site (Figure 1B). The *Hry* deletion shows incomplete penetrance with *Hry/+* mice being phenotypically normal except that some have a white belly spot. The *Hry/Hry* mice on the other hand have megacolon, growth impairment, and loss of pigmentation. The 15.9-kb deleted region contains three evolutionary conserved regions, denoted *Sox10-MCS7*, -8, and -9, with *Sox10-MCS7* as the most promising candidate for being a *Sox10* enhancer. In a follow up study, this element was studied using reporter constructs in transgenic zebrafish and was confirmed to act as an enhancer element in melanocytes (Antonellis et al., 2008). The *Sox10-MCS7* segment was shown to direct a broad reporter gene expression in almost all tissues where *Sox10* is normally expressed and together with the deletion of the *Sox10-MCS8* and 9 it can thus explain the severe defects in the *Hry* mouse. Thus, the mouse *Sox10-MCS7* element is likely to play a role in the expression of *Sox10* during the migration and development of melanocytes. Interestingly, the *Sox10-MCS7* element in mouse is homologous to the conserved non-coding sequence that is deleted in *DB* chickens (Figure 1B).

The demonstration that *Sox10-MCS7* is an important *Sox10* enhancer in mice suggests that the deletion causing the *DB* phenotype in chickens may constitute a *cis*-acting regulatory mutation affecting *SOX10* expression. However, there is a marked phenotypic difference between *Hry* mice and *DB* chickens, where the *Hry* causes strong pleiotropic effects, including megacolon and defects in melanocyte migration, whereas the effect of the *DB* mutation appears to be restricted to more subtle changes in differentiated melanocytes; a large number of fully viable chickens are homozygous *DB/DB*. One possible explanation for this difference is that the *Hry* deletion encompasses three highly conserved elements (*MCS7*, -8, and -9), whereas the *DB* deletion only affects the *MCS7* homolog. Furthermore, species differences as regards the functional role of the homologous sequence as well as differences in pigment cell biology between birds and mammals may also play

a role. One possible scenario for the mechanism of action for the *DB* mutation is that the deletion of the *MCS7* homolog reduces *SOX10* expression which in turn down-regulates the expression of key enzymes for melanin production, such as tyrosinase and *DCT*, either directly or indirectly by reducing the expression of *MITF* that together with *SOX10* has a critical role in regulating pigment synthesis in vertebrates (Levy et al., 2006). Such a scenario would be consistent with data from the mouse showing that *Sox10* can regulate the expression of melanocyte-specific genes including *Tyr* and *Dct* (Hou and Pavan, 2008). The expected consequence of a reduced expression of tyrosinase is that the production of red pheomelanin is favored, which is the characteristic feature of the *DB* phenotype. This hypothesis should now be tested by analyzing the expression of *SOX10*, *MITF* and possible downstream targets in melanocytes from different genotypes at the *DB* locus.

The *DB* phenotype is an interesting model for plumage color variation because it affects the pattern of plumage color rather than causing a general pigmentation defect. It is very likely that a majority of the variation of plumage color we observe between species are caused by this type of regulatory mutations causing spatiotemporal changes in the expression of pigmentation genes. An interesting aspect of the *DB* phenotype is that *DB* males (and to some extent *DB* females) show a stronger pheomelanistic expression in the ventral than the dorsal parts of the chicken with the male tail being not reddish at all. One possibility is that this pattern occurs because the deleted regulatory element is more important in melanocytes in breast feathers than in tail feathers. Another possible scenario is that this pattern is a result of the dose relationship between *agouti* (*ASIP*) and *MC1R*, with *MC1R* expression being down-regulated in *DB* birds. Dorsoventral expression differences of *ASIP* mRNA isoforms have recently been found in quail (Nadeau et al., 2008), and an expression difference of *MC1R* in the *DB* individuals might therefore lead to a dorsoventral pigmentation difference in the birds because of the normal pattern of *ASIP* expression. In *DB* birds, the relationship between *ASIP* and *MC1R* could then come below a threshold in the breast (dorsal) part, but not in the tail (ventral) part. This can be investigated by analyzing the expression patterns of *SOX10*, *MITF*, *MC1R*, *ASIP*, *TYR*, and *DCT* as well as the neighboring *PICK1* gene that may potentially be affected by the deletion.

Methods

An intercross between the obese strain (*OS*) of White Leghorn chicken (Cole, 1966) and the red junglefowl (*RJF*) was generated by crossing two *RJF* males with eight *OS* females and one *OS* male with two *RJF* females. From the *F*₁ generation, eight males and 35 females were selected to generate an *F*₂ generation comprising 756 individuals. The study has been approved by the local

Ethical Committee of the Swedish National Board for Laboratory Animals. High-quality digital pictures were taken of all the *F*₂ birds every third week of their life (at 1, 3, 6, 9, 12, 15, 18, 21, 24, and 28 weeks of age). All pictures were sorted by individual in Extensis Portfolio 6 (Extensis, Inc, Portland, OR, USA) and used for phenotypic classification of the birds.

DNA samples from different breeds with and without the *DB* allele collected by the AvianDiv project (Hillel et al., 2003) were used for resequencing and association analysis across breeds.

The methods used for linkage analysis, genotyping and sequencing are described in the Appendix S1. The primers used for resequencing the *DB* region are described in Table S2.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Methods.

Table S1. Summary of resequencing the 12.8-kb fragment upstream of *SOX10* associated with the Dark brown phenotype in chicken.

Table S2. Primer sequences.

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