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Sex-linked barring in chickens is controlled by the *CDKN2A/B* tumour suppressor locus

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Summary

Sex-linked barring, a common plumage colour found in chickens, is characterized by black and white barred feathers. Previous studies have indicated that the white bands are caused by an absence of melanocytes in the feather follicle during the growth of this region. Here, we show that Sex-linked barring is controlled by the *CDKN2A/B* locus, which encodes the INK4b and ARF transcripts. We identified two non-coding mutations in *CDKN2A* that showed near complete association with the phenotype. In addition, two missense mutations were identified at highly conserved sites, V9D and R10C, and every bird tested with a confirmed Sex-linked barring phenotype carried one of these missense mutations. Further work is required to determine if one of these or a combined effect of two or more *CDKN2A* mutations is causing Sex-linked barring. This novel finding provides the first evidence that the tumour suppressor locus *CDKN2A/B* can affect pigmentation phenotypes and sheds new light on the functional significance of this gene.

Introduction

The diversity of pigmentation in both natural populations and domesticated animals is one of the most studied traits in biology. Pigmentation diversity became a subject for scientific studies in the beginning of the 20th century after the rediscovery of Mendelian genetics (Bateson, 1902; Haldane et al., 1915). Hundreds of genes have been discovered that influence pigmentation in a range of species. In just the mouse, 159 genes affecting pigmentation have been reported (Montoliu et al., 2009). Many of the described causative mutations alter the coding sequence and thus have been straightforward to pinpoint. However, the genetic basis for the bewildering diversity of pigmentation patterns among species, particularly among birds, is poorly understood and is unlikely to be determined by simple loss-of-function mutations.

Plumage colour and patterning show great variation among breeds of chickens. To date, five loci controlling

Significance

The *CDKN2A/B* locus has a key role in cell cycle regulation. It encodes both the ARF protein, which binds the p53-stabilizing protein MDM2, and the INK4 protein, a cyclin-dependent kinase inhibitor. Loss-of-function mutations in *CDKN2A* are responsible for familiar forms of human melanoma. Our study establishes a new animal model for functional studies of *CDKN2A* because it provides conclusive evidence that mutation(s) in this gene underlies the Sex-linked barring plumage colour in chickens. A barred feather pattern is very common among birds, and to our knowledge, this is the first time that a gene controlling such a pigmentation pattern has been identified.

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Figure 1. (A) A Barred Plymouth Rock adult rooster (*B*B/B*B*) showing the Sex-linked barring feather pattern, where white bars are approximately twice the width of black bars. (B) A number 2 under major tail covert feather (counting from medial to distal) taken at 8 weeks of age from a *B*B* homozygous male (top) showing considerably wider white bands compared to a feather taken from a *B*B/W* hemizygous female (bottom). (C) At hatch, Barred Plymouth Rock chicks display a white spot on their heads. The *B*B/W* female (left) has a smaller spot compared to the *B*B/B*B* male (right). (D) Peregrine falcon exhibiting barred feathers. Photos A, B and C supplied by Christa F. Honaker and photo D by Jorma Luhta/naturepl.com.

plumage colour have been characterized at the molecular level (Chang et al., 2006; Gunnarsson et al., 2007; Kerje et al., 2003, 2004; Vaez et al., 2008). Two particularly interesting phenotypes involving the pattern of pigmentation are Sex-linked barring (Figure 1) and autosomal barring (Crawford, 1990). The difference in appearance of these barring patterns is that Sex-linked barring is characterized by bars with complete absence of pigmentation whereas autosomal barring produces alternating bars with darker and lighter pigmentation resulting in a more irregular pattern. Most likely due to its camouflaging properties, a barring pattern is found in the wild-type plumage colour of many avian species, exemplified by the peregrine falcon in Figure 1D. The two genetically controlled barring patterns in chickens provide excellent opportunities to study the genetic mechanisms underlying this biologically important phenotype. Sex-linked barring shows a monogenic inheritance whereas autosomal barring is assumed to be caused by the combined effect of alleles at the *Dark brown* (*Db*) and *Pencilling* (*Pg*) loci (Crawford, 1990).

In birds, females are the heterogametic sex (Z/W) and males the homogametic sex (Z/Z). Sex-linked inheritance of *Sex-linked barring* (*B*B*) and the wild-type (*B*N*) was reported as early as 1908 (Spillman, 1908). Here, we use the official chicken genetics nomenclature where the first letter (*B*) refers to the locus and the second letter (*B* or *N*) refers to the allele. Bitgood (1988) assigned the *B* locus to the q-arm of chromosome Z by linkage mapping, and recently it was mapped to a 355 kb-region on the distal q arm of this chromosome (Dorshorst and Ashwell, 2009). The *B* locus shows a classical gene dosage effect: *B*B* homozygotes display wider white bands than either *B*B/B*N* heterozygotes or *B*B/W* hemizygotes (Figure 1B), and in doing so illustrates that dosage compensation is not well developed in birds (Melamed and Arnold, 2007). Other characteristic phenotypic effects of the *B* allele include the dilution of the dermal pigment in the shanks and beak and a white spot on the head which is larger in males than females (Figure 1C). The spot on the head can be used to determine the sex of chicks at hatch (Jerome, 1939).

During the 1920s and 1930s, researchers first attempted to explain the mechanisms underlying Sex-linked barring. Danforth and Foster (1929) speculated that the pattern could be caused by a rhythmic variability in metabolism or hormonal secretions (i.e. that the Sex-linked barring factor should be present in the skin). They performed skin transplants between one-day old barred and non-barred chickens and observed that skin transplanted from one sex to the other grew feathers that, in structure, were like those of the host and, in colour and barring pattern, like those of the donor. Montalenti (1934) observed that two adjacent barring feathers did not necessarily develop the white and black bands simultaneously. Together, these two studies indicate that a gene product causing barring must act in the feather follicle.

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Nickerson (1944) and Bowers and Asano (1984) reported premature melanocyte death in Barred Plymouth Rock chicken (BPR) feather follicles at the time of white band development. This is a breed in which the *B* allele is fixed. Both authors suggested that cell death was the cause of the white bands on barred feathers. Subsequently, Bowers (1988) proposed that white bands formed due to an autophagic degeneration of the melanocytes at the proximal edge of a black band in the feather follicle. During the formation of the white bands of a BPR feather, few, if any, melanocytes were observed, and cell culture experiments showed that BPR feather melanocytes died approximately five times earlier than wild-type feather melanocytes. More recently, the same group reported that melanocytes from sex-linked barred feathers were more sensitive to oxidative stress than those of wild-type chickens (Bowers et al., 1994). They proposed that in Sex-linked barring individuals, melanocytes die due the high levels of reactive oxygen species (ROS) present during pigment production, and when ROS levels decrease, pigmentation progresses normally until there is again an increase in ROS levels. The width of the white bands is dependent on both the growth rate of the feather and if the bird is homozygous or heterozygous at the *B* locus. Nickerson (1944) calculated the mean developmental time for white bands in male BPR to be 5–6 days.

In this study, we show that Sex-linked barring is caused by the *CDKN2A/B* locus, also known as the *INK4b-ARF* locus (Figure 2). In humans, *CDKN2B* encodes the INK4b transcript and *CDKN2A* encodes two transcripts, INK4a and ARF (Figure 2). Each of the three transcripts has its own promoter. The *CDKN2A/B* locus has been shown to be involved in tumour suppression and cell proliferation as ARF and INK proteins regulate the cell cycle (Quelle et al., 1995). INK4 inhibits cyclin-D-dependent kinases, causing a cell cycle arrest in the G1 phase. In both the humans and mice, the ARF-p53-MDM2 network either regulates the cell cycle and transition from G1 to S-phase or directs the cell to apoptosis.

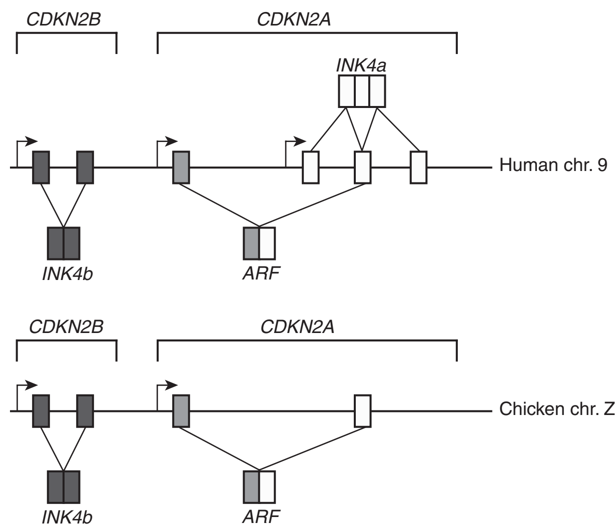

Figure 2. Schematic figure of the *CDKN2B-CDKN2A* locus. (A) In humans, *CDKN2B* encodes the INK4b transcript and *CDKN2A* encodes the INK4a and ARF transcripts. (B) In the chicken, the INK4a transcript is absent and the ARF coding sequence is considerably shorter than the human homolog.

Kim et al. (2003) reported the absence of the INK4a transcript in chickens and suggested that the tumour suppressor function was compensated for by other genes. Chicken INK4b appears to have the same functions as typical INK4 proteins as demonstrated by transfection experiments where chicken INK4b coprecipitated with human Cdk4 and Cdk6. Chicken INK4b also caused substantial inhibition of cell proliferation. A phylogenetic comparison places chicken INK4b between mammalian INK4a and INK4b, suggesting that the INK sequence was duplicated in mammals, rather than that one of the copies was subsequently lost in chickens (Kim et al., 2003).

Although chicken ARF is considerably shorter than mammalian ARF due to a stop codon in the beginning of exon 2, it shares many properties with mammalian ARF. Chicken ARF coimmunoprecipitates with MDM2 (mouse double minute 2 homolog) just like human ARF, and chicken ARF can protect p53 from MDM2-mediated destruction. Kim et al. (2003) suggested that the additional mammalian ARF residues encoded by exon 2 were not important for function, but rather were acquired by alterations in the splicing register.

Based on linkage mapping and high-resolution identical-by-descent (IBD) mapping we can now present conclusive evidence that the Sex-linked barring phenotype in the chicken is controlled by the *CDKN2A/B* locus.

Results

Linkage analysis

The *CDKN2A/B* locus was identified by linkage analysis as a positional candidate gene for *Sex-linked barring*. The analysis was performed using 675 F_2 chickens in a three-generation intercross between one red junglefowl (RJF) male (*B*N/B*N*) and three White Leghorn females (*B*B/W*). The highly significant LOD scores (LOD >25) obtained between *Sex-linked barring* and markers at the distal end of chromosome Zq (Table 1), were consistent with previously published data (Dorshorst and Ashwell, 2009). The multi-point analysis placed *Sex-linked barring* in the interval between the Single Nucleotide Polymorphism (SNP) markers M1 (chrZ:72,805,560 bp) and M4 (chrZ: 73,551,809 bp). However, this region of the genome is poorly assembled and contains gaps between contigs. We developed two additional SNP markers M2 (chrZ:73,433,470 bp in contig 154) and M3

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Table 1. Linkage map of distal end of Zq-arm including *Sex-linked barring*. The region from M2 to CDKN2A/B (in bold) are oriented in the opposite direction to that reported for the 2006 assembly

Marker	Position in assembly (bp)	Position in linkage map (cM)	LOD-score versus Sex-linked barring
rs16121677	65 351 508	148.1	8.7
rbl12118	71 175 861	163.8	20.1
M2	**73 433 470**	**176.6**	**38.4**
rbl179	**73 147 455**	**177.8**	**82.1**
M1	**72 805 560**	**180.1**	**46.6**
M3	**72 492 150**	**181.3**	**27.4**
Sex-linked barring	**–**	**183.2**	**–**
CDKN2A/B	**72 009 897**	**183.2**	**47.3**
M4	73 551 809	190.7	44.1
rbl73	73 657 362	195.4	31.6
rbl82	73 765 055	199.4	22.8
rbl111	74 084 933	212.4	9.5
M5	74 367 110	222.0	2.1

(chrZ:72,492,150 bp in contig 174) which were found by resequencing. Linkage analysis using these markers revealed that contigs 154 and 174 should be oriented in the direction opposite of the one given in the May 2006 UCSC assembly (Table 1). The region flanked by the M3 and M4 markers harbours nine known genes; *Methylthioadenosine phosphorylase (MTAP), Cyclin-dependent kinase inhibitor 2 (CDKN2A/B), Tripartite motif-containing 36 (TRIM36), Protein geranyltransferase type I, beta subunit (PGGT1B), Coiled-coil domain containing 112 (CCDC112), Fem-1 homolog c (FEM1C), Aldehyde dehydrogenase family 7, member A1 (ALDH7A1), GRAM domain containing 3 (GRAMD3),* and *Thioredoxin-like 1 (TXNL1)*. *CDKN2A/B* is an an attractive candidate gene for *Sex-linked barring* because loss-of-function mutations in this gene are associated with familiar forms of melanomas in humans, demonstrating an important functional role in melanocyte biology (Dracopoli and Fountain, 1996; Hussussian et al., 1994; Kannengiesser et al., 2007). A new SNP marker located within the *CDKN2A/B* locus was therefore developed. No recombination event was detected between the *CDKN2A/B* marker and *Sex-linked barring*, as would be required if the *CDKN2A/B* locus controlled *Sex-linked barring* (Table 1). The linkage to the *CDKN2A/B* locus was supported by a LOD score of 47.3.

The assignment of *Sex-linked barring* to this region on chromosome Zq was confirmed using the intercross between the Obese strain (OS) of chickens (*B*B*) and the RJF (*B*N*). However, the recombination frequency was much lower in this cross and no recombinant was observed in the interval between markers rs16121860 (chrZ:65,566,460 bp) and M4 (chrZ:73,551,809 bp).

IBD mapping reveals complete association between a 12 kb *CDKN2A/B* haplotype and *sex-linked barring*

An identical-by-descent (IBD) region is expected when all individuals carrying *Sex-linked barring* share the same causative mutation. A number of *B*B* and *B*N* homozygotes were therefore resequenced for the candidate *CDKN2A/B* locus. All *B*B* birds shared the same *CDKN2A/B* haplotype whereas the *B*N* birds showed a high degree of haplotype diversity, suggesting this region harboured *Sex-linked barring*. To define the size of the IBD region among individuals carrying the *B*-allele, six *B*B* and 20 *B*N* birds were sequenced up- and downstream of the identified IBD region (Figure 3). This revealed a minimal shared haplotype of

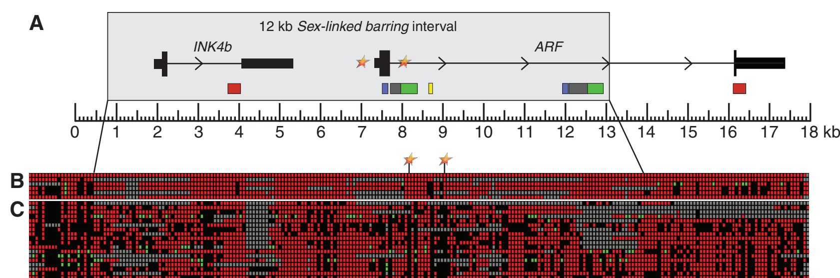

Figure 3. Identical-by-descent (IBD) mapping of *Sex-linked barring*. (A) The 12 kb region in complete association with *Sex-linked barring* is indicated in grey. This region was found to contain duplicated (red, blue, and green boxes) and highly repetitive (yellow box) sequences. The repetitive segment contained a 75 bp deletion event, which was fixed in all Sex-linked barring individuals investigated (n = 6). Of the non-barred individuals tested (n = 30), only the Brown line individuals (n = 12) were found to be segregating for this indel. Yellow stars indicate the positions of SNP1 and SNP2 which are closely associated with *Sex-linked barring*. (B) Alignment of SNP data for the IBD region in the reference sequence (line 1: a male bird from the White Leghorn Obese strain, *B*B2/B*B2*) and individuals representing five additional breeds assumed to be fixed for *Sex-linked barring* (lines 2–6: Järhöns, *B*B2/B*B2*; Broiler Low line, *B*B1/B*B1*; Barred Plymouth Rock, *B*B1/B*B1*; Coucou de Rennes, *B*B1/B*B1* and White Leghorn Line 13, *B*B0/W*). SNPs are colour coded with respect to the reference sequence (line 1) as follows: red, identical to the reference; green, heterozygous; black, homozygous or hemizygous for the opposite allele to the reference and grey, missing data (C) Data representing populations presumed fixed for the wild-type allele (*B*N*) colour coded as before. Lines 1–5, red junglefowl; line 6–7: grey junglefowl, Ceylon junglefowl; line 8–10: Icelandic chickens; line 11–17: Yurlow, Brown line, Bourbonnaise, Marans, Orlov, Poltava Clay, Smyth line and line 18: red junglefowl reference sequence from the May 2009 chicken genome assembly (http://www.genome.ucsc.edu).

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12 kb (at reference position 912–13 160 bp in the submitted GenBank sequence GU470992) spanning *INK4b* and the coding region of *ARF* (Figure 3). The IBD region is remarkably small but consistent with the very high recombination rate at the distal end of chicken chromosome Zq (Groenen et al., 2009). For comparison, the IBD region associated with the *Greying with age* mutation in horses was 352 kb (Rosengren Pielberg et al., 2008). These results provide strong genetic evidence that the causative mutation(s) is located within the 12 kb shared region. In addition, to the duplications (blue and green boxes) at the *CDKN2A/B* locus reported by Kim et al. (2003), one duplication (red box) and one highly polymorphic repeat region (dark grey box) were identified in this study (Figure 3). However, none of the repeat polymorphisms were associated with the Sex-linked barring phenotype.

The minimal shared haplotype contained 320 SNP positions. Two SNPs in non-coding DNA appear to be exclusively associated with Sex-linked barring. The first, SNP1, is within the *CDKN2A* (*ARF*) promoter region, 265 bp upstream of the transcription start site (according to GenBank AY138245), and the second, SNP2, is located at nucleotide position 385 bp in *CDKN2A* intron 1 (Figure 3). SNP2 is found in one of the duplicated regions indicated by a green box in Figure 3. SNP1 and SNP2 were genotyped in 285 individuals representing 51 populations to measure the strength of association across populations (Table 2). We found that 97 presumed *B*B* homozygotes representing 16 populations were homozygous for the alleles associated with *Sex-linked barring* at both SNPs. Because *Sex-linked barring* is dominant, the observation of any of the two SNP alleles associated with *Sex-linked barring* in an individual with a reliably scored wild-type phenotype would exclude that SNP as a causative mutation. However, nearly all presumed *B*N* birds were homozygous for the alternate alleles at SNP1 and SNP2 (Table 2). The only exceptions to this were one Icelandic chicken and one RJF from a Swedish zoo population (see below).

There were two additional polymorphisms exclusively associated with *Sex-linked barring* but these were polymorphic amongst *B* haplotypes. The two SNPs occur in the coding region of *CDKN2A*, at positions 7500 bp and 7502 bp, in the reference sequence (GenBank GU470992), and are missense mutations: V9D and R10C (Figure 4). They could be functionally important as both residues are highly conserved among vertebrates (Figure 4), and it is well established that this part of the ARF protein is of crucial functional importance (di Tommaso et al., 2009; Kim et al., 2003; Moulin et al., 2008). We decided to denote the *Sex-linked barring* allele carrying the V9D substitution *B1* because its association with the Sex-linked barring phenotype is well established by the phenotypic appearance of BPR (Figure 1A), Coucou de Rennes, and Coucou du Vercors chickens. Similarly, the allele carrying the R10C substitution was denoted *B2* because its association with Sex-linked barring was established in our study based on segregation data from intercrosses involving White Leghorn Line 13 and the Obese line (derived from White Leghorn). Moreover, we also identified an allele identical to *B1* and *B2* for the entire 12 kb IBD region except that it has neither the V9D nor the R10C missense mutation (Figure 4). We denote this allele *B0* as it is closely related to *B1* and *B2*. Whilst *B0* was found in the White Leghorn Line 13 line which is presumed to be fixed for *Sex-linked barring*, its association to the phenotype could not be evaluated. This was due to both the masked phenotypic expression by *Dominant white* and because there were no segregation data for *B0*. Although one of the founder females (P7) in our intercross between White Leghorn Line 13 and RJF was hemizygous *B*B0/W*, its Z chromosome was not transmitted to any of the F_1 birds used in the next generation. Further sequence analysis of the two presumably non-barred birds, one RJF and one Icelandic chicken, homozygous for the SNP1 and SNP2 alleles associated with *Sex-linked barring* showed that they should also be classified as *B0/B0* because they did not carry either missense mutation.

To exclude the possibility that the causative mutation for *Sex-linked barring* was due to a chromosomal rearrangement (e.g. a duplication event), a Southern blot experiment was performed using two probes targeting *CDKN2B* and *CDKN2A* respectively. Genomic DNA from ten Barred and ten non-barred chickens was digested with *Bam*H1. The blot revealed identical fragment patterns and no obvious differences in hybridization intensities (data not shown). Thus, there was no indication that the causative mutation constitutes a chromosomal rearrangement.

SNP1 and SNP2 in CDKN2A are located in putative E2F-1 and NFκB transcription factor binding sites

SNP1 at position 7064 (A → G) in our reference sequence (GenBank GU470992), 256 bp upstream of the transcription start site and within the promoter region of *CDKN2A*, was in near complete association with the *B*-alleles. Possible transcription factor binding sites (TFBS) were predicted using the Consite software (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite) (Lenhard et al., 2003). A putative E2F-1 TFBS created by the SNP associated with *B*B* was predicted (score 4.9), whilst the *B*N*-allele prediction score was slightly lower (score 4.2). E2F-1 is known to regulate *CDKN2A* expression in both mouse (Lomazzi et al., 2002) and chicken cell lines (Kim et al., 2006). The same prediction analysis was performed using the region surrounding SNP2 at position 385 in intron 1 of *CDKN2A*. The *B*N* sequence was predicted to better match a NFκB motif (score 4.6) than the *B*B* sequence (score 2.9).

Electrophoretic mobility shift assays (EMSA) were used to investigate possible differences in transcription

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Table 2. Genotype results for SNP1 and SNP2 at the *CDKN2A* locus for a large set of diverse populations

	SNP 1 (promoter)			SNP 2 (intron1)		
Breed	A/A	A/G	G/G	C/C	A/C	A/A
Domestic chicken, barred						
Barred Plymouth Rock	0	0	1	0	0	1
Broiler (France)[1]	0	0	8	0	0	10
Broiler (France)[1]	0	0	8	0	0	8
Broiler (Israel)[1]	0	0	8	0	0	3
Broiler Dam[1]	0	0	1	0	0	1
Cochin	0	0	0	0	0	1
Coucou de Rennes	0	0	6	0	0	1
Coucou du Vercors	0	0	5	0	0	5
Dorking	0	0	0	0	0	1
Järhöns	0	0	8	0	0	6
White Leghorn, commercial[1]	0	0	7	0	0	7
White Leghorn, line 13[1]	0	0	1	0	0	1
White Leghorn, Obese strain[1]	0	0	20	0	0	13
White Plymouth Rock	0	0	21	0	0	18
White egg layer[1]	0	0	1	0	0	3
Domestic chicken, heterozygous barred						
Owl Bearded	0	2	0	0	2	0
Domestic chicken, non-barred						
Australorp	5	0	0	0	0	0
Bedouin	1	0	0	0	0	0
Black Minorca	1	0	0	0	0	0
Bourbonnaise	8	0	0	2	0	0
Brown-egg layer	1	0	0	1	0	0
Brown Leghorn	1	0	0	0	0	0
Brown Line	2	0	0	2	0	0
Cochin	8	0	0	2	0	0
Czeck Golden Pencilled	5	0	0	0	0	0
Dorking	4	0	0	1	0	0
Fayoumi	5	0	0	5	0	0
Finnish Landrace	5	0	0	1	0	0
Friesian fowl	5	0	0	0	0	0
Godollo	1	0	0	0	0	0
Green-legged Partridge	1	0	0	1	0	0
Houdan	5	0	0	0	0	0
Icelandic	14	0	1[2]	11	0	1[2]
Marans	8	0	0	6	0	0
Noir de Janzé	1	0	0	1	0	0
Orlov	1	0	0	1	0	0
Owl Bearded	8	0	0	1	0	0
Padovana	1	0	0	0	0	0
Poltava Clay	8	0	0	3	0	0
Red Villafranquina	5	0	0	0	0	0
Rhode Island Red (France)	8	0	0	0	0	0
Rhode Island Red (The Netherlands)	8	0	0	0	0	0
Label chickens, slow-growing line	12	0	0	0	0	0
Smyth Line	2	0	0	2	0	0
Transylvanian Naked Neck	5	0	0	1	0	0
Ukranian bearded	1	0	0	0	0	0
Westfälischer Totleger	4	0	0	0	0	0
Yurlow	4	0	0	4	0	0
Junglefowl, non-barred						
Ceylon junglefowl	4	0	0	2	0	0
Grey junglefowl	4	0	0	1	0	0
Red junglefowl	30	0	1[3]	22	0	1[3]
Total	186	2	97	70	2	81

[1]These birds are white and presumed to be fixed for *Sex-linked barring*.
[2]A bird with light plumage, which makes it difficult to phenotype.
[3]Presumed to be non-barred but no detailed phenotypic record or photo is available.

factor binding between the alleles at the SNP1 and SNP2 loci using nuclear extracts from mouse melan-a cells and oligonucleotides corresponding to both allelic variants. No DNA-protein complex was detected with the oligonucleotides corresponding to the two SNP1 alleles, while a control probe with a perfect E2F-1 binding site (Parisi et al., 2002) gave a specific complex using the same experimental conditions (Figure S1A). A specific complex was obtained when oligonucleotides corresponding to both alleles at the SNP2 site were used (Figure S1B). The migration of the obtained complex was different from the most prominent complexes detected using a control oligonucleotide representing an established NFκB binding site (Chen et al., 1999), suggesting that another protein or complex of several proteins may bind the chicken oligonucleotide (Figure S1B). However, there was no obvious difference in the results obtained with the oligonucleotides corresponding to the two SNP2 alleles.

Luciferase reporter assays were performed for SNP1 and SNP2 to test whether alleles at these loci influenced transcriptional activation. The constructs were transfected into mouse melan-a cells. Reporter constructs corresponding to both SNP1 alleles gave significantly higher luciferase activities than the control reporter (pGL3 Basic vector) showing that whilst the region has promoter activity as expected, there were no significant differences between alleles (Figure S1C). Similarly, constructs corresponding to both alleles at SNP2 significantly enhanced luciferase expression compared to an empty pGL3 Promoter construct, but again there was no significant difference between alleles (Figure S1D). Cotransfection with the E2F-1 transcription factor and the constructs harbouring the *CDKN2A* (*ARF*) promoter confirmed the results of Kim et al., 2006 that transcription of chicken *CDKN2A*, like mammalian *CDKN2A*, is activated by E2F-1, although there is no significant difference in activity between the SNP1 alleles (Figure S1E). When constructs containing all four combinations of SNP1 and SNP2 alleles were tested for possible interactions between SNPs, no sig-

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Species/Population	Allele	ARF residue 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Chicken																	
Red junglefowl	N	M	T	S	R	I	R	C	T	V	R	L	R	R	A	R	S
White Leghorn, line 13	B0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Barred Plymouth Rock	B1	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.
Broiler Low line	B1	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.
Broiler High line	B1	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.
Coucou de Rennes	B1	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.
Coucou du Vercors	B1	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.
White Leghorn, Obese strain	B2	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
White Leghorn, line 13	B2	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
White Leghorn, commercial	B2	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
Järhöns	B2	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
Zebra finch	-	.	.	P	.	.	.	.	.	I	H	F	.	.	.	.	.
Human	-	.	V	R	.	F	L	V	.	L	.	I	.	.	.	C	G
Rhesus monkey	-	.	V	R	.	F	L	V	.	L	.	I	.	.	.	C	G
Mouse	-	.	G	R	.	F	L	V	.	.	.	I	Q	.	.	G	R
Dog	-	.	V	R	A	F	L	I	.	.	.	O	.	.	.	G	R
Rat	-	.	G	R	.	F	V	V	.	.	.	I	.	.	T	G	R
Opossum	-	.	V	R	.	V	.	V	.	.	.	V	S	.	.	C	R

Figure 4. Multiple sequence alignment of amino acids 1–16 of the ARF protein. The allele denoted *B1* is firmly associated with *Sex-linked barring* based on phenotypic data. The allele denoted *B2* is firmly associated with *Sex-linked barring* based on segregation analysis. The allele denoted *B0* shares the 12 kb identical-by-descent region in *CDKN2A* with alleles *B1* and *B2*; however, lacks the missense mutations in codon 9 and 10. *B0* was found in a population presumed to be fixed for *Sex-linked barring*, although an association with the Sex-linked barring phenotype has not been confirmed. Sequences from other species are derived from Ensembl (http://ensembl.org/index.html).

nificant differences in luciferase activity were detected (Figure S1F).

Discussion

This study demonstrated that *Sex-linked barring* in chickens is controlled by the *CDKN2A/B* locus. This conclusion is based on the complete association between *Sex-linked barring* and a 12 kb haplotype encompassing the INK4b transcript and part of the ARF transcript. Within this haplotype we identified two non-coding SNPs, one in the promoter region and one in intron 1 of *CDKN2A* that showed nearly complete association to the phenotype. Furthermore, two different missense mutations were identified in the ARF transcript, V9D and R10C, and these were exclusively associated with two different *Sex-linked barring* haplotypes, named *B1* and *B2*, respectively. That these two missense mutations occur at two residues that are highly conserved among vertebrates further supports our conclusion that this locus is controlling *Sex-linked barring*.

Arf knock-out mice do not show an altered coat colour (Sviderskaya et al., 2002), suggesting that the *CDKN2A* mutation(s) causing *Sex-linked barring* is not a complete loss-of-function, but rather a gain-of-function or regulatory mutation. It is not yet clear which of the above mentioned mutations or alternatively which combination of two or more mutations is underlying this phenotype. One possibility is that *Sex-linked barring* is genetically heterogeneous and that the two missense mutations have very similar effects on ARF function and cause a similar or identical phenotype. Every chicken we tested that exhibited the barring phenotype carried one of these two missense mutations. A scenario where the missense mutations are causative and the non-coding SNPs have no effect is possible, but less likely because the two missense mutations occurred on the same *CDKN2A/B* haplotype. Our genetic screen showed that this haplotype is rare or absent among wild-type birds. An alternative hypothesis is that one of the two non-coding SNPs, or the combined effect of the two causes Sex-linked barring and that the two missense mutations have no effect. This explanation is very unlikely given the high degree of sequence conservation of the mutated residues associated with *Sex-linked barring*. Furthermore, we observed two presumably non-barred chickens that were homozygous for the alleles at the non-coding SNPs associated with *Sex-linked barring*. Because segregation data are not available from this rare haplotype, we cannot formally exclude the possibility that it has a phenotypic effect and that they represent phenotype misclassifications. We favour a scenario where an initial non-coding mutation, SNP1, SNP2 or a combination of the two, resulted in a visible phenotype, and that the two subsequent missense mutations were independently selected because they enhanced the phenotypic effect. This suggests a model where either of the two missense mutations are required but may not be sufficient to cause Sex-linked barring.

The classical Sex-linked barring phenotype could be caused by the combined effect of two or more mutations. Examples where two subsequent mutations underlie a pigmentation phenotype in domestic animals include Dominant white colour in pigs (Marklund et al., 1998), black-spotting in pigs (Kijas et al., 1998), Smoky in chickens (Kerje et al., 2004) and white spotting in dogs (Karlsson et al., 2007). This hypothesis can be tested as we identified the *B0* haplotype that carries

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the variant alleles at the two non-coding SNP1 and SNP2 loci but is wild-type for the two missense mutations at *CDKN2A* codon 9 and 10. We have been unable to observe phenotypic effects associated with the *B0* haplotype because it occurs in a line (White Leghorn Line 13) fixed for *Dominant white*, which masks the expression of *Sex-linked barring*. However, breeding experiments can be designed to generate *B0* progeny lacking the *Dominant white* allele to test the phenotypic effect of this haplotype.

Our attempts to study the functional significance of the two non-coding polymorphisms in *CDKN2A* using EMSAs and luciferase assays did not reveal significant differences between any allele pair. This does not exclude that one or both of these SNPs are functionally important. These assays were performed using mouse melan-a melanocytes, cells that do not perfectly replicate the regulatory network operating in feather follicle melanocytes. It is possible that a putative transcription factor interacting with these sites does not exist in the mouse, or that the chicken and mouse homologs have diverged to the extent that they do not bind the same recognition sites. Further studies of these polymorphisms would be justified if the above mentioned breeding experiments provide conclusive data that they are functionally significant.

Previous studies concerning the development of feather pigmentation in BPR chickens have indicated that *Sex-linked barring* causes premature melanocyte cell death (Bowers and Asano, 1984; Nickerson, 1944). We now show that this phenotype is caused by a mutation(s) in the locus encoding CDKN2A, a protein with a well-established role for regulating cell proliferation and cell death of melanocytes. Loss-of-function mutation in *CDKN2A* is a major risk factor for familial forms of melanoma in humans (Goldstein et al., 2008; Randerson-Moor et al., 2001). Furthermore, Sviderskaya et al. (2002) reported that the expression of Ink4a-Arf in mice causes melanocyte senescence and stimulates pigment synthesis. A plausible mechanism for *Sex-linked barring* in chickens is that the *CDKN2A* mutation(s) we identified results in premature cell death, which in turn leads to the formation of white bars lacking melanocytes. This may then be followed by a new wave of melanocytes recruited from a pool of stem cells, which migrate, colonize the feather follicle, produce melanin and form the next black bar. Thus, the mutations causing Sex-linked barring in chickens may have an opposite effect than mutations associated with familiar forms of melanoma in humans (i.e., premature cell death versus reduced cell death).

This is the first study demonstrating that mutations in the tumour suppressor locus *CDKN2A/B* can underlie variation in coat or plumage colour. It will therefore be of considerable interest to study the possible role for this locus in determining the bewildering colour diversity among species, particularly among birds. That *CDKN2A/B* is sex-linked in birds and does not show dosage compensation, suggests that this locus may contribute to the remarkable sexual dimorphism in avian plumage colour. For instance, the zebra finch is named after the zebra striped feathers exhibited only by the males and *CDKN2A/B* is an obvious candidate locus contributing to this spectacular sexual dimorphism. Interestingly, according to the recently released zebra finch genome assembly (http://www.genome.ucsc.edu), its *CDKN2A/B* gene encodes an ARF protein that like the one encoded by the chicken *B2* allele differs from other vertebrate ARF sequences by not having an arginine residue at position 10 (Figure 4).

Materials and methods

Animals

Linkage analysis was performed using a three-generation intercross between one RJF male *B*N/B*N* and three White Leghorn (WL) *B*B/W* females (Schutz et al., 2002). The results were confirmed using an intercross between the OS of chickens and the RJF. The OS line was established in 1955 from an experimental WL population and constitutes a well-established animal model for autoimmune thyroiditis (Cole, 1966). Two RJF males were crossed with eight OS females and one OS male with two RJF females. In the F_1 generation, eight males and 35 females were selected to generate an F_2 generation of 756 individuals. The DNA samples used for IBD-mapping and SNP genotyping are summarized in Table 2.

Linkage analysis

Linkage analysis was performed using the CRIMAP software version 2.4 (Green et al., 1990) and SNPs previously typed in the two crosses (Wahlberg et al., 2007). Additional SNPs added to the distal end of chromosome Z to map *Sex-linked barring* were genotyped using pyrosequencing and PyroGold chemistry (Biotage, Uppsala, Sweden). PCR and sequencing primers are listed in Table S1.

Identical-by-descent mapping

The *CDKN2A/B* locus was resequenced in six homozygous barred *B*B* and 18 non-barred *B*N* individuals. The region was amplified by long range PCR and sequenced with internal primers. Three highly repetitive segments were cloned (Fermentas, Canada) and sequenced multiple times in order to discern the true sequence of both alleles. All primers were designed using the PRIMER3 software version 0.4.0 (Rozen and Skaletsky, 2000). DNA sequences were analyzed and edited with CODONCODE ALIGNER 2.0.4. (CodonCode, Dedham, MA, USA).

Southern blot analysis

Southern blot analysis was performed using two probes targeting *CDKN2B* and *CDKN2A* (Table S1). Genomic DNA extracted from ten barred and ten non-barred individuals was digested with the restriction enzyme *Bam*H1 and separated by 0.7% agarose gel electrophoresis.

Transcription factor binding site analysis and EMSA

Transcription factor binding site analysis was performed using CONSITE (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite) (Lenhard et al., 2003). Nuclear extracts from normal murine melanocytes, melan-a cells (Bennett et al., 1987), were prepared using the Nuc-

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Buster Protein Extraction kit (Novagen, Madison, WI, USA). Electrophoretic mobility shift assays were performed as previously described with minor modifications (Van Laere et al., 2003). The oligonucleotides were annealed in 1× NEB2 buffer (New England Biolabs, Ipswich, MA, USA) and 10 μg of nuclear extracts from melan-a cells were preincubated on ice for 20 min in binding buffer (Markljung et al., 2009) for SNP2 EMSA. Competition reactions were supplemented with 4000 fmol (100 × molar excess) unlabelled ds-oligonucleotide. After the addition of 40 fmol end-labelled ^{32}P-dCTP ds-oligonucleotide, reactions were incubated at room temperature for 30 min. Complexes were separated by electrophoresis on a 1.5 mm 5% native 29:1 polyacrylamide gel in 0.5× TBE at 70 V for 3–4 h.

Cell culture

The melan-a mouse melanocyte cell line was cultured at 37°C in a humidified atmosphere of 5% CO_2 using RPMI 1640 (Gibco, Karlsruhe, Germany) supplemented with 10% foetal bovine serum (FBS; BioWest, Miami, FL, USA), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich), 200 pM cholera toxin (CT; Sigma-Aldrich) and 200 nM phorbol 12-myristate 13-acetate (TPA; Sigma-Aldrich). Cholera toxin and TPA are essential for the proliferation and survival of normal melanocytes in cell culture. Cultures were split approximately every 4–5 d.

Luciferase assay

The regions containing *CDKN2A* SNP1 and SNP2 were generated with PCR using KOD Hot Start DNA Polymerase (Novagen, USA). The luciferase reporter constructs were made by cloning the two alleles at both SNPs into the pGL3 Basic vector (SNP1) or pGL3 Promoter vector (SNP2; Promega, Madison, WI, USA). Constructs containing different combinations of SNP1 and SNP2 alleles were made by cloning the regions into pGL3 Basic. The pCMV6-E2F-1 vector expressing mouse E2F-1 was purchased from Origene (Rockville, MD USA). Melan-a cells were transfected at 90% confluency in 6-well plates. Two microgram of Luciferase reporter plasmid and 50 ng of control *Renilla* plasmid phRG-Basic (Promega) were transfected into each well using 4 μl Lipofectamine 2000 CD reagent (Invitrogen, Carlsbad, CA, USA). For cotransfection experiments, 1, 20, 50 or 100 ng pCMV6-E2F-1 were added to previously described transfection reactions for SNP1. Transfections were performed in opti-MEM (Gibco, Germany). Cells were harvested 24 h post-transfection, and Firefly and *Renilla* activities were measured using the Dual Luciferase Reporter Assay System (Promega) and an Infinite M200 Luminometer (Tecan Munich GmbH, Germany). Luciferase values were divided by *Renilla* values to normalize for fluctuations in plated cell amounts and efficiency between transfections. Expression values for all test constructs were compared to the expression of the empty vector.

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Supporting information

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

Figure S1. (A) Electrophoretic mobility shift assay (EMSA) showing absence of specific binding to SNP1 barring (B) or wild-type (N) ds-oligonucleotide. The last four lanes show binding to a probe harbouring a perfect E2F-1 binding site (P_E). (B) EMSA showing absence of significant differences in binding to SNP2 barring (B) or wild-type (*N*) oligonucleotides. The last lane shows binding to an oligonucleotide harbouring a perfect NFκB binding site (P_N). (C) Luciferase assay of reporter constructs containing SNP1 sequence corresponding to either the barred (*B*) or wild-type (*N*) allele and the empty vector pGL3-Basic. (D) Luciferase assay of reporter constructs containing SNP2 sequence corresponding to either the barred (*B*) or wild-type (*N*) allele and the empty vector pGL3-Promoter. (E) Luciferase assay of reporter constructs containing SNP1 sequence corresponding to either the barred (*B*) or wild-type (*N*) and the empty vector pGL3-Basic as well as a construct with non-ARF test-DNA. Cotransfection with 1–100 ng of E2F-1. (F) Luciferase assay of reporter constructs containing a combination of the (*B*) and (*N*) alleles in the same plasmid. The reporter construct with only the barring allele is shown as a reference, as well as the empty vector pGL3-Basic. Values on the *y*-axis show the Relative Luciferase Units (RLU): Firefly reporter luciferase levels in relation to control Renilla luciferase levels.

Table S1. Oligonucleotides used in amplification, sequencing, genotyping and electrophoretic mobility shift assay experiments related to Sex-linked barring in chicken.

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