

# Genetic mapping of the sex-linked barring gene in the chicken

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**ABSTRACT** The sex-linked barring gene of the chicken (*Gallus gallus*), first identified in 1908, produces an alternating pattern of white and black bars in the adult plumage. More recent studies have shown that melanocytes in the developing feather follicle of the Barred Plymouth Rock experience premature cell death, whereas initially it was thought that melanocytes remained viable in the region of the feather devoid of pigmentation but were simply inhibited from synthesizing melanin. In an attempt to reconcile these 2 different hypotheses at the molecular level, we have taken a gene mapping approach to isolate the sex-linked barring gene variant. We developed a mapping population consisting of 71 F<sub>2</sub> chickens from crossing a single Barred Plymouth Rock female with a White Crested Black Polish male. Existing and novel microsatellite markers located on the chicken chromosome Z were used to genotype all individuals in our mapping population. Single marker

association analysis revealed a 2.8-Mb region of the distal q arm of chicken chromosome Z to be significantly associated with the barring phenotype ( $P < 0.001$ ). Further analysis suggests that the causal mutation is located within a 355-kb region showing complete association with the barring phenotype and containing 5 known genes [micro-RNA 31 (*miRNA-31*), methylthioadenosine phosphorylase (*MTAP*), cyclin-dependent kinase inhibitor 2B (*CDKN2B*), tripartite motif 36 (*TRIM36*), and protein geranylgeranyltransferase type I,  $\beta$  subunit (*PGGT1B*)], none of which have a defined role in normal melanocyte function. Although several of these genes or their homologs are known to be involved in processes that could potentially explain the barring phenotype, our results indicate that further work directed at fine-mapping this region is necessary to identify this novel mechanism of melanocyte regulation.

**Key words:** Barred Plymouth Rock, sex-linked barring, feather pigmentation, melanocyte

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## INTRODUCTION

The Barred Plymouth Rock (BPR) chicken (*Gallus gallus*), with its characteristic pattern of alternating white and black bars of feather pigmentation, is perhaps the most recognizable breed displaying the sex-linked barring (*B*) phenotype. Although the exact origin of the *B* variant is unknown, the BPR breed is believed to have been developed from crosses involving the Black Java, Black Cochon, and Dominique breeds in America during the mid 19th century. Barring was identified as a sex-linked gene by Spillman (1908), one of the first examples of a sex-linked trait reported after the initial demonstration of Mendelian principles of inheritance in animals (coincidentally, also done using poultry) by Bateson in 1902 (Bateson, 1902; Dodgson, 2003; Siegel et al., 2006). At this time, it was recognized that *B* represented a dominant pigment-inhibiting gene inherited on a sex chromosome, displayed on a genetically black feather pigment background in the case of the BPR breed (Punnett, 1923). Later, *B* was shown to be 13.7

cM from inhibitor of dermal melanin (*Id*) on the long arm of *Gallus gallus* chromosome Z (**GGZ**) (Bitgood, 1988). Studies using random amplified polymorphic DNA markers have placed *Id* at 210 cM on the genetic map (Levin et al., 1993), further corroborating the location of *B* on the distal end of the q arm of GGZ.

The biogenesis of melanin is a complex multistep process involving the conversion of L-tyrosine to 5,6-dihydroxyindole by the rate-limiting enzyme tyrosinase (Ito and Wakamatsu, 2008) and results in the production of potentially cytotoxic compounds (Urabe et al., 1994). Melanin is produced in melanosomes within the melanocyte and is transferred through dendritic cellular processes to the keratinocytes that make up the growing feather in the feather follicle of the chicken (Yu et al., 2004). In the chicken, feather pigmentation defects have been linked to tyrosinase (*TYR*), solute carrier family 45, member 2 (*SLC45A2*), melanocortin 1 receptor (*MC1R*), and melanocyte protein 17 precursor (*PMEL17*) (Kerje et al., 2003, 2004; Chang et al., 2006; Gunnarsson et al., 2007). Mutations in these genes can result in the complete gain or loss of pigmentation across all feathers of the chicken, giving insight into the broader molecular mechanisms governing pigmentation in the feather. However, the genetic

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mechanisms controlling the distribution of intrafeather pigmentation may shed further light on the processes of melanocyte differentiation and function in relation to the interaction between the melanocyte and keratinocyte of the growing feather. The plethora of pigmentation patterns in the chicken provides an opportunity to probe the many different points at which the gene networks governing pigment cell development may be disrupted.

Some of the earliest experiments directed at unraveling the cellular biology of the barring variant involved embryonic ectodermal tissue transplants between barred and unpigmented breeds of poultry. These experiments showed that the barred pigmentation pattern defect is autonomous to the melanocyte (Willier, 1941) and that the periodicity of the white and black bars is characteristic of the donor (Nickerson, 1944). Transplants of unpigmented melanocytes from the boundary between a black and newly forming white region of a barred feather to an unpigmented host are capable of producing pigment. This suggested to Nickerson that melanocytes carrying the barring defect are sensitive to the build up of a diffusible inhibitor of pigment synthesis that must be cleared from the region of active feather growth before pigmentation production can continue. More recent experiments indicate that melanocytes on the interface between black and white bars show an increased level of enzymes indicative of autolysis (Bowers, 1988); however, results from this experiment contradict Nickerson's results in that melanocytes were found to be absent from unpigmented areas of the feather. In vitro studies showed that *B* melanocytes are capable of surviving and producing pigment in culture for 30 d in a similar fashion to wild-type melanocytes except for a decrease in melanosome number in the *B* melanocytes (Bowers and Gatlin, 1985). A decrease in melanocyte and melanosome number was also observed in the choroid tissue of the BPR as compared with Jungle Fowl, with melanosomes in the BPR-derived melanocytes forming abnormal clumps in the melanocyte cytoplasm (Schreck and Bowers, 1989).

The hypotheses of a diffusible inhibitor of melanin synthesis put forth by Nickerson (1944) or that of premature cell death ascribed to by Bowers (1988) are valid given the current suite of knowledge on feather morphogenesis and melanoblast differentiation. Previous experiments have clearly shown that a defect in melanocyte function is responsible for the alternating cycles of pigmentation production in the barred feather. However, the observation of markers indicative of premature cell death is not overly informative of the causal mutation of the barring defect but speaks to a general disruption of normal melanocyte function. The diffusible inhibitor hypothesis involves a cessation of pigment production in an otherwise viable melanocyte until such a time as the inhibitor can be cleared and pigmentation production can once again begin. The premature cell death hypothesis relies upon a supply of melanoblasts, presumably from the extrafollicular der-

mis, that repopulate the feather blastema at defined periodic intervals and differentiate into pigment-producing melanocytes (Lucas and Stettenheim, 1972). A paucity of recent work directed at unraveling the molecular mechanisms of the barring defect and resolving the discrepancies between the viability of melanocytes in regions of the feather containing white barb ridges leads us to take a classical forward genetics approach to mapping the genomic region associated with this pigmentation trait. The overall objective of identifying the causal mutation of the barring phenotype in the chicken is directed at broadening our understanding of the interaction between the melanocyte, melanosome, and keratinocyte, key functional elements of feather pigmentation morphogenesis.

## MATERIALS AND METHODS

An F<sub>2</sub> mapping population was derived from a single BPR female and White Crested Black Polish male. A single F<sub>1</sub> male was mated to 7 full-sibling females, which resulted in 71 F<sub>2</sub> chicks. Typically, chicks carrying the dominant *B* allele are recognizable at hatch as those with a white streak on the head on an otherwise black chick down background. However, in this cross, the white crest contributed by the male founder individual masked the head streak phenotype. For this reason, phenotypes were collected on the F<sub>2</sub> progeny at 12 wk of age as third generation feathers displaying the adult feather pattern replaced solid-colored juvenile feathers. Both founders of the mapping population carried the dominant black allele at the extended black locus (*E*), which confers black pigmentation to the feather except in the presence of the dominant allele at the *B* locus. The identical *E* locus genetic background allowed for birds to be easily distinguished as barred or black. All experimental procedures involving birds were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Genotyping was performed using conventional dye-labeled microsatellite markers located on GGZ. Existing markers were screened on the 2 founder individuals and if determined to be fully informative (male homozygous for different alleles than the hemizygous female), the complete population consisting of 81 individuals was genotyped for the corresponding marker. A single SNP (rs16765615) in the *B* candidate gene melan-A (*MLA-NA*) was genotyped by dye terminator sequencing of a PCR fragment generated using 5'-CCTTGCAT-GTTTCGTGTCAT-3' and 5'-TGAAACAGATGCA-CAGTCAGC-3' primers. After the first scan of GGZ with known microsatellite markers and rs16765615, putative tandem repeats were chosen from the genome database University of California, Santa Cruz Chicken Genome Browser May 2006 assembly (Kent et al., 2002) that were located in the regions showing the highest level of association with the phenotype. These putative microsatellite markers were developed using a M13 universal dye-labeled scheme (Schuelke, 2000) in which

the forward primer contains an 18-bp M13 primer sequence 5' to the fragment-specific sequence to facilitate screening of a large number of potentially informative markers with a single dye-labeled M13 primer.

Standard PCR reactions contained 2.5 mM MgCl<sub>2</sub>, 0.2 mM deoxynucleoside triphosphate, 1 μM forward and reverse primers, 1X MasterAmp PCR Enhancer with Betaine (EpiCentre, Madison, WI), 1X PCR Gold Buffer (ABI, Foster City, CA), 0.375 U of AmpliTaq Gold DNA Polymerase (ABI), and 50 ng of genomic DNA in 15-μL total volume. The M13 universal primer PCR reactions were the same except that they contained 1 μM M13 universal dye-labeled primer, 1 μM reverse primer, and 0.25 μM forward primer. Standard PCR thermal cycling parameters consisted of an initial denaturation step at 95°C for 5 min, 30 cycles of 95°C, a marker-specific annealing temperature, and 72°C for 30 s each followed by a final elongation step at 72°C for 5 min. The M13 universal primer PCR thermal cycling conditions were the same except for 12 cycles of 95°C, 53°C, and 72°C, for 30 s each, inserted before the final elongation step to allow for incorporation of the M13 universal dye-labeled primer. The PCR products were analyzed by capillary electrophoresis on an ABI PRISM 3100 Genetic Analyzer (ABI) and PCR fragment size was determined using GeneMapper Software v3.7 (ABI). Primer sequences for new markers have been deposited in GenBank with the accession numbers GF099533-GF099545 and GF100456-GF100459.

A genetic linkage map was constructed using the FIXED option in CriMap (Green et al., 1990) based on the physical order of the markers in the genome sequence database. The FLIPS option was used to assess

the likelihood of the current map as compared with permutations involving flanking markers. A single marker association analysis was performed using PLINK (Purcell et al., 2007) and *P*-values were adjusted for multiple testing using false discovery rate (Benjamini and Hochberg, 1995). Although we expect complete association between closely linked markers and the *B* locus due to the dominant mode of inheritance and complete penetrance of the phenotype, a significance threshold of the corrected *P*-values was set at *P* < 0.001 to define broader regions significantly associated with the trait.

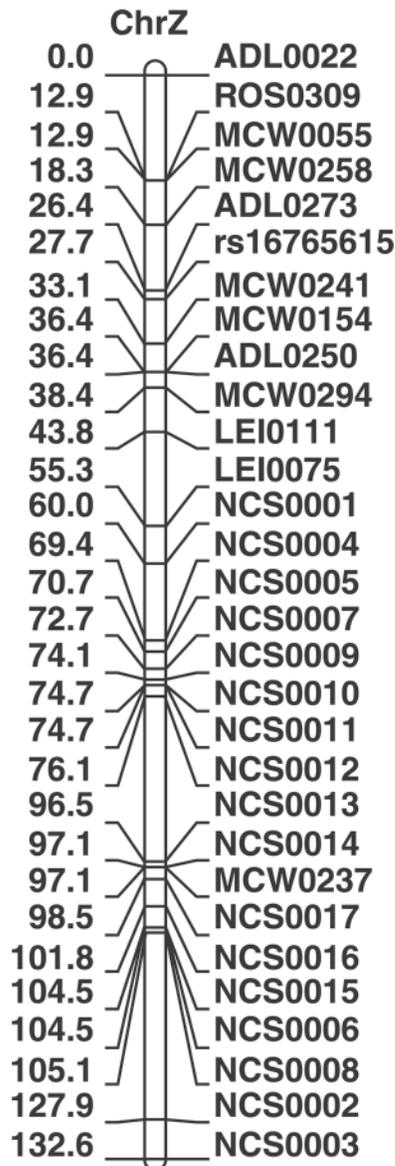
## RESULTS

Of the 71 F<sub>2</sub> birds that were phenotyped at 12 wk of age, 17 were barred males, 15 were black males, 21 were barred females, and 18 were black females. Individuals from the F<sub>2</sub> generation that represent the 2 alternative feather patterns are shown in Figure 1. The known inhibiting effect of the *B* allele on shank pigmentation (Hutt, 1949; Smyth, 1990) is clearly evident in the barred individual in Figure 1. The segregation of *Id* in this population, which is also a strong inhibitor of shank pigmentation and is located 13.7 cM from *B* on GGZ (Bitgood, 1988), magnifies the difference in pigmentation between shanks of barred and black individuals. However, no data were collected on shank pigmentation due to the difficulty of accurately distinguishing between the effects of *B* and *Id* when both genes are segregating within a population.

The genetic map constructed from the 30 markers genotyped in this F<sub>2</sub> cross (Figure 2) encompasses 132.6 cM, spanning over 94% of the 74.6-Mb GGZ and rep-



**Figure 1.** Feather pigmentation pattern of 2 representative individuals from the F<sub>2</sub> generation of the mapping population. A black female is shown on the left and a barred female is shown on the right.



**Figure 2.** The genetic linkage map of chromosome Z (ChrZ) in centimorgans (left) for 30 genetic markers (right).

resents an average recombination rate of 1.78 cM/Mb. The order of markers in the genetic map was based on the May 2006 assembly of the physical genome sequence database. The FLIPS option in CriMap indicated that minimal gains in the likelihood of genetic marker order would be achieved by flipping a small number of closely linked markers. Due to the limited number of recombination events between the markers in question and their location on the map (isolated from the *B* region of interest), we retained the marker order from the physical genome assembly. In comparison to a recently generated high-density SNP linkage map of the GGZ (Wahlberg et al., 2007), our genetic map displays a smaller total map length and average recombination rate, most likely due to incomplete detection of all recombination events by our smaller set of genetic markers. The increase in recombination rate (cM/Mb) at the extreme distal end of the q arm of GGZ shown in Figure 3 was also observed by Wahlberg et al. (2007),

although we were unable to detect several other recombination hotspots identified in their study due to lower marker density.

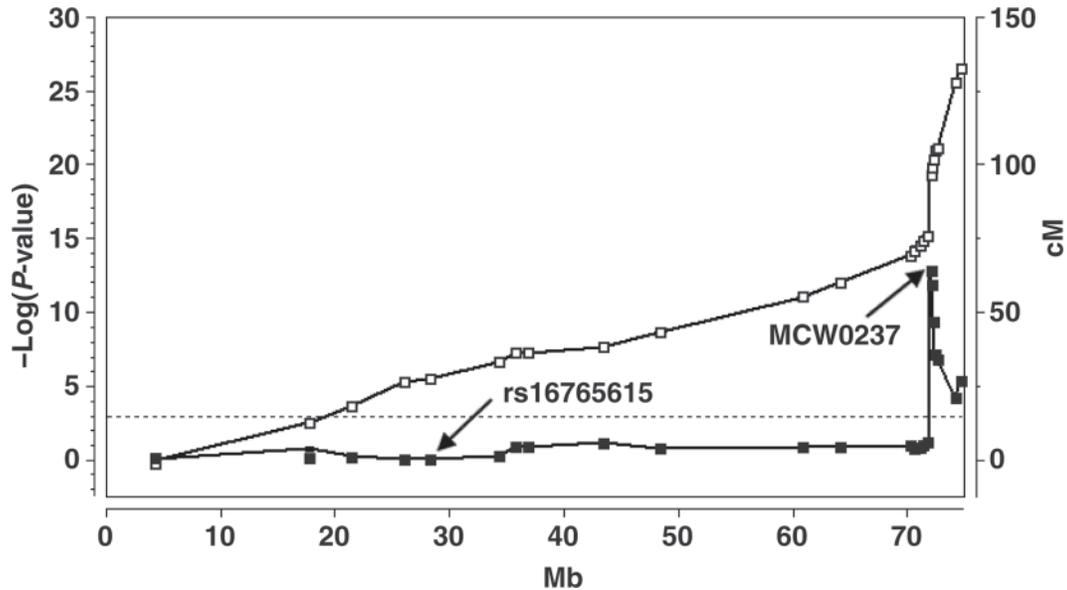
The SNP rs16765615 was included in this study because of its location in the barring candidate gene *MLANA*. The gene *MLANA* is required for proper processing and trafficking of *PMEL17* in the melanocyte (Hoashi et al., 2005) and a 3-amino acid insertion within *PMEL17* has been associated with another feather pigmentation variant in the chicken, dominant white (Kerje et al., 2004). Our analysis showed that *MLANA* is not associated with the barring feather pigmentation pattern despite the known interaction between *MLANA* and *PMEL17* (Figure 3).

However, the association analysis did reveal that the marker MCW0237 located at 72 Mb was highly associated with the barring phenotype. Initially, MCW0237 was isolated by 12 Mb from the nearest existing microsatellite marker (LEI0075), which showed no association with the trait. With the addition of novel microsatellite markers, the region showing significant association to the barring phenotype was refined to the distal 2.8 Mb of the q arm of GGZ (Figure 3). With the identification of 2 markers that show complete linkage with the barring phenotype (NCS0014 and MCW0237) flanked by additional markers showing incomplete linkage (NCS0012 and NCS0017), we have defined the region containing *B* as the 355-kb sequence from 71,799,096 to 72,154,034 bp on GGZ (Figure 4). We do not define NCS0013 as the left boundary of the region of interest due to the single incidence of incomplete linkage of this marker with the barring phenotype occurring as an event of double recombination between NCS0012 and NCS0014.

## DISCUSSION

Our results show that although more than 2.8 Mb of the distal end of GGZ show a significant association with the barring phenotype, the 355-kb region between NCS0012 and NCS0017 is the most probable location of the barring variant. This 355-kb region harbors 5 known genes and 5 predicted genes (known genes are shown in Figure 4). Gene ontology of the 5 known genes [micro-RNA 31 (*miRNA-31*), methylthioadenosine phosphorylase (*MTAP*), cyclin-dependent kinase inhibitor 2B (*CDKN2B*), tripartite motif 36 (*TRIM36*), and protein geranylgeranyltransferase type I,  $\beta$  subunit (*PGGT1B*)] in the 355-kb region reveals no direct link to melanocyte-specific processes. Functional attributes of *miRNA-31* remain unknown, leaving the role that this noncoding element plays in the barring phenotype open.

Tripartite motif 36 (*TRIM36*) is a member of the RING finger, B-box type zinc finger, and coiled-coil domain gene family that is involved in a wide variety of cellular processes including viral response, exocytosis, apoptosis, transcriptional regulation, cell cycle regulation, keratinocyte differentiation, and neurite outgrowth (Meroni and Diez-Roux, 2005). Localization of

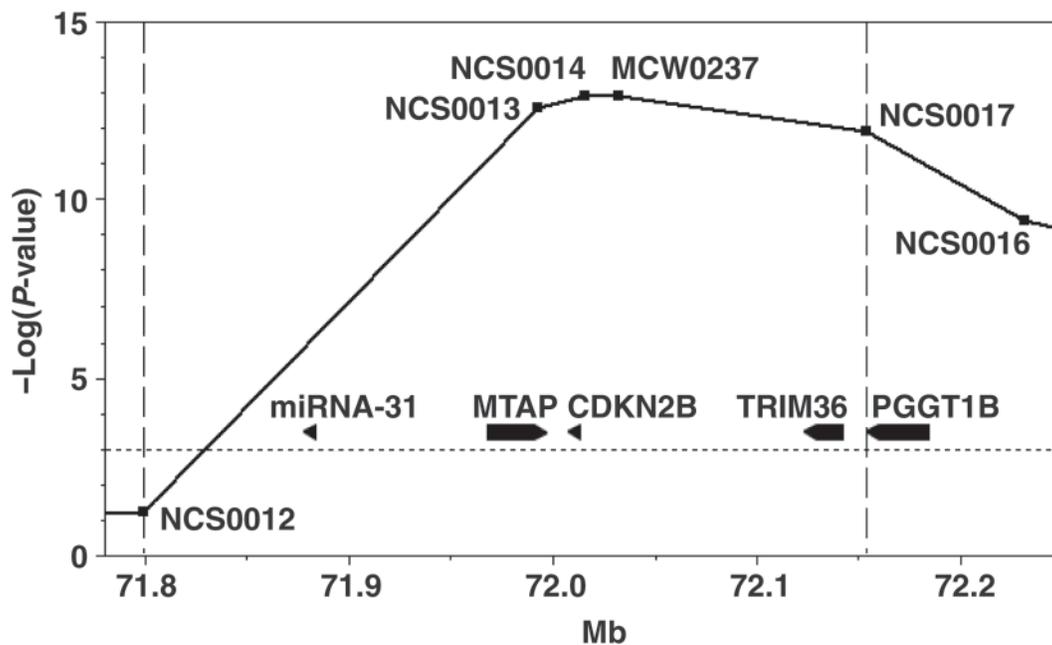


**Figure 3.** *P*-values for marker by barring phenotype association test (left y-axis, ■-) and genetic map distance (right y-axis, -□-) by physical map distance (x-axis) along the entire chromosome Z. The region of highest association with the barring phenotype can be seen at approximately 72 Mb as well as an increase in the recombination rate (cM/Mb) at the distal end of the chromosome. The dashed line represents the significance threshold of  $P < 0.001$ . Marker rs16765615 in the candidate gene melan-A does not show significant association with the barring phenotype, whereas MCW0237 is within the region of highest association.

*TRIM36* to the microtubule cytoskeleton (Short and Cox, 2006) suggests that it may play a role in vesicle transport, a process that is necessary for melanosome trafficking in properly functioning melanocytes. However, *TRIM36* has only been shown to be expressed in haploid testicular germ cells and is known to be in-

involved in the acrosomal exocytosis process (Kitamura et al., 2005).

Protein geranylgeranyltransferase type I,  $\beta$  subunit (*PGGT1B*) is a member of the prenyltransferase family of proteins that direct the lipid modification of acceptor proteins characterized by specific amino acid mo-



**Figure 4.** The region showing complete linkage with barring phenotype defines a 355-kb region of interest on chicken chromosome Z (GGZ). All markers above the horizontal dashed line (representing  $P < 0.001$ ) show a significant association with the barring phenotype. The markers NCS0014 and MCW0237 are in complete linkage with the barring phenotype and NCS0013 and NCS0017 show nearly complete linkage with the barring phenotype. The marker NCS0012 is not significantly associated with the trait and defines the left boundary of the region of interest. The marker NCS0017, while significantly associated, is not in complete linkage with the barring phenotype and thus defines the right boundary of the region of interest (demarcated by vertical dashed lines). Known genes found within the region of interest are shown. miRNA-31 = micro-RNA 31; MTAP = methylthioadenosine phosphorylase; CDKN2B = cyclin-dependent kinase inhibitor 2B; TRIM36 = tripartite motif 36; PGGT1B = protein geranylgeranyltransferase type I,  $\beta$  subunit.

tifs at the C-terminus. Prenylation defects have been shown to have an effect on intracellular targeting, processing, and function of acceptor proteins that regulate lysosomal-related organelle function, such as the melanosome (Gomes et al., 2003). A mutation in the splice acceptor site of *PMEL17* in the gunmetal mutant mouse has been shown to result in reduced geranylgeranylation of Rab27 in platelets. The gunmetal mouse is named for a dilution of coat color in conjunction with platelet defects resembling a variation of Hermansky-Pudlak syndrome in humans. However, the mechanism of the causal mutation has not been directly investigated in the melanosome other than to note the presence of the abnormally spliced transcript of Rab geranylgeranyl transferase  $\alpha$  (*RABGGTA*) (Detter et al., 2000). The ashen mouse also exhibits a dilution in coat color in conjunction with platelet defects found to be caused by a mutation in *Rab27a*, the downstream target of defective *RABGGTA* identified in the gunmetal mouse (Wilson et al., 2000); Rab27a is known to associate with myosin Va and melanophilin to facilitate the process of melanosome translocation to the cell periphery before the transfer of melanin to the keratinocyte can occur (Seabra and Coudrier, 2004; Raposo et al., 2007). These studies suggest that the process of geranylgeranyl modification of key proteins in the organelle receptor machinery complex is necessary for proper melanosome trafficking inside the developing melanocyte and that *PGGT1B* may have an unexplored role in this process. Investigation of *PGGT1B* expression revealed that it is expressed in the feather pulp of both barred and black birds (unpublished data). However, the marker NCS0017, corresponding to a 16-bp insertion/deletion mutation in the 3' untranslated region of *PGGT1B*, displays less than complete linkage with the barring phenotype. With the majority of *PGGT1B* located outside of the 355-kb region of interest identified in this study, it is unlikely that it corresponds to *B*, although the possibility of a more 3' regulatory mutation still remains.

In the chicken, *CDKN2B* has a similar function to that of cyclin-dependent kinase inhibitor 2A (*CDKN2A*) as documented in other vertebrate species (Kim et al., 2006), with *CDKN2A* being absent in the chicken genome. Methylthioadenosine phosphorylase (*MTAP*) and *CDKN2A* are associated with malignant melanoma (Monzon et al., 1998; Borg et al., 2000; Behrmann et al., 2003); however, deletions of these 2 genes are found in many malignant cell types (Stadler and Olopade, 1996; Dreyling et al., 1998; Schmid et al., 1998; Ishii et al., 1999; M'Soka et al., 2000), suggesting that these genes may have a broader role in cell proliferation. However, recent work with the gray coat color variant in the horse has demonstrated that a *cis*-acting regulatory mutation in syntaxin 17 (*STX17*) alters expression of nuclear receptor subfamily 4, group A, member 3 (*NR4A3*) and its downstream target cyclin D2 (*CCND2*) (Pielberg et al., 2008). The increased expression of *CCND2* is hypothesized to increase the rate of melanocyte pro-

liferation and subsequently exhaust the population of melanocyte precursors of the hair prematurely while allowing rampant melanocyte growth in the skin. Over the life span of the horse, this results in graying of the hair as melanocytes are obligated to undergo cell death as the hair is formed while melanocytes in the skin vigorously persist and develop into melanomas. The gene *CDKN2B* may play a similar role in the barring phenotype of the chicken by altering the melanocyte cell cycle in a manner that depletes the actively growing regions of the feather follicle of functional melanocytes prematurely. The repopulation of the feather blastema by extrafollicular melanocyte stem cells, if occurring in a regulated and periodic fashion, could explain the alternating regions of pigmentation in the feather.

We have determined that the causal mutation of the sex-linked barring feather pigmentation pattern is within a 355-kb region on GGZ containing a limited number of genes and a single noncoding element that have not been previously implicated in the process of pigmentation production. The lack of known melanocyte effector genes in the region we have identified illustrates the potential usefulness of historically described chicken pigmentation mutants in uncovering novel mechanisms of melanocyte regulation. Although the link between any of the genes in the 355-kb region and a specific causal mechanism for sex-linked barring remains weak, *PGGT1B* and *TRIM36* support the diffusible inhibitor hypothesis put forth by Nickerson (1944) via their potential association with melanosome transport and the necessary cytoskeleton components for this process. The role of *CDKN2B* in cell cycle regulation clearly fits with the premature cell death hypothesis developed by Bowers (1988) due to the highly important role of cyclins in this process. We have made significant progress toward identifying the specific genetic mechanism behind this pigmentation variant and are continuing this work with the development of an F<sub>3</sub> generation for fine-mapping purposes.

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