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Targeted analysis of four breeds narrows equine Multiple Congenital Ocular Anomalies locus to 208 kilobases

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Abstract The syndrome Multiple Congenital Ocular Anomalies (MCOA) is the collective name ascribed to heritable congenital eye defects in horses. Individuals homozygous for the disease allele (MCOA phenotype) have a wide range of eye anomalies, while heterozygous horses (Cyst phenotype) predominantly have cysts that originate from the temporal ciliary body, iris, and/or peripheral retina. MCOA syndrome is highly prevalent in the Rocky Mountain Horse but the disease is not limited to this breed. Affected horses most often have a Silver coat color; however, a pleiotropic link between these phenotypes is yet to be proven. Locating and possibly isolating these traits would provide invaluable knowledge to scientists and breeders. This would favor maintenance of a desirable coat color while addressing the health concerns of the affected breeds, and would also provide insight into the genetic basis of the disease. Identical-by-descent mapping was used to narrow the previous 4.6-Mb region to a 264-kb interval for the *MCOA* locus. One haplotype common to four breeds showed complete association to the disease (Cyst phenotype, $n = 246$; MCOA phenotype, $n = 83$). Candidate genes from the interval, *SMARCC2* and *IKZF4*, were screened for polymorphisms and genotyped, and segregation analysis allowed the MCOA syndrome region to be shortened to 208 kb. This interval also harbors *PMEL17*, the gene causative for Silver coat color. However, by shortening the *MCOA* locus by a factor of 20, 176 other genes have been unlinked from the disease and only 15 genes remain.

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Introduction

Multiple Congenital Ocular Anomalies (MCOA) syndrome is a congenital nonprogressive syndrome described in horses. The most frequent feature of this disease is fluid-filled cysts of variable sizes (2–20 mm) in the posterior iris and ciliary body epithelium within the eye. Two distinct ocular phenotypes exist: (1) cysts that originate from the posterior iris, temporal ciliary body, and/or peripheral retina (Cyst phenotype), and (2) cysts in combination with additional ocular defects including iridocorneal angle

abnormalities, cornea globosa, iris hypoplasia, congenital cataracts, lens subluxation, focal areas of retinal detachment, microphthalmia, and macropalpebral fissures (MCOA phenotype). Horses with MCOA have abnormal pupillary light reflexes and pupils do not dilate after administration of mydriatic drugs (Ramsey et al. 1999a, b). Individual MCOA-affected horses may or may not have the complete set of congenital defects described above. Both the distinct subdivision of phenotypes and the transmission of the disease within our pedigrees are consistent with a mutant allele displaying incomplete dominance. The Cyst horses are heterozygous and have an intermediate phenotype compared to the horses with multiple anomalies that carry two copies of the disease allele (Ewart et al. 2000; Andersson et al. 2008).

Conditions have been favorable for the mutation causing MCOA to be enriched in the Rocky Mountain Horse breed (Ewart et al. 2000). This breed originated from a very limited number of founder horses, which were used extensively to develop the breed. In fact, many Rocky Mountain horses can be traced back to a single foundation stallion. The horses within the breed have been selected for a distinctive four-beat gait and the Silver coat color has been highly favored. The fact that an intensive selection process can lead to amplification of undesirable traits has been demonstrated in several other horse breeds [e.g., hyperkalemic periodic paralysis (Rudolph et al. 1992), hereditary equine regional dermal asthenia (Tryon et al. 2007), severe combined immunodeficiency (Shin et al. 1997), and Overo Lethal White Syndrome (Santschi et al. 1998)]. In the Rocky Mountain Horse breed, selection of horses with the highly desirable Silver coat color has simultaneously increased MCOA syndrome as these traits are linked on horse chromosome 6 (Andersson et al. 2008). The Silver coat color in horses is characterized by dilution of black pigment in the hair and revealed to be associated with a missense mutation in *pre-melanosomal protein 17* or *PMEL17* (Brunberg et al. 2006). Additional horse breeds that have been diagnosed with MCOA include the Kentucky Mountain Saddle Horse, Mountain Pleasure Horse (both closely related to the Rocky Mountain Horse), Belgian Draft, Morgan Horse, Shetland Pony, American Miniature Horse (Ramsey et al. 1999a; Grahn et al. 2008; Komaromy and Rowlan 2009), and the Icelandic Horse (B. Ekesten, unpublished).

Cysts are found in most affected horses and are usually bilateral. Horses with cysts usually have normal functional vision irrespective of cyst size since cysts are either translucent or lightly pigmented. A small number of juvenile horses that have cornea globosa as a component of multiple ocular defects have a considerable refractive error and subsequently poor vision. This refractive error is corrected by emmetropization as the juvenile horse eye achieves adult size (Ramsey et al. 2000).

The primary objective of this research was to identify the gene and mutation that regulate the MCOA syndrome in horses. The results would have practical implications such as enable genetic testing for precise diagnosis of carrier horses and horses with the multiple eye defects. We previously mapped the locus for equine MCOA to a 4.9-Mb interval on horse chromosome 6 by linkage mapping (Andersson et al. 2008). In the present study we have significantly limited the interval for the *MCOA* locus and selected two additional candidate genes in the interval, *IKZF4* and *SMARCC2*, for mutation screening. *IKZF4* was selected as a candidate gene because it is expressed in ocular tissue, is a member of the IKAROS family of transcription factors, and interacts with the gene microphthalmia-associated transcription factor, *MITF* (Hu et al. 2007). *MITF* influences both pigmentation and ocular development (Hodgkinson et al. 1993). Mutations in *MITF* can cause Waardenburg syndrome type 2A (Nobukuni et al. 1996), an auditory-pigmentary disorder that affects ocular development. In addition, a mutation in the *PAX3* gene causes Waardenburg syndrome type 1, and it has been suggested that it occurs through a failure to regulate *MITF* (Watanabe et al. 1998). The other candidate gene, *SMARCC2*, was selected because it encodes a 170-kDa protein that is one of 12 proteins in the SWI/SNF complex (Dechassa et al. 2008). The ATPase component *Brm* of this complex regulates differentiation of early retinal stem cells (Das et al. 2007). In humans, the SWI/SNF chromatin remodeling complex is involved in the regulation of the *CRYAB* ($\alpha\beta$-crystallin) gene that is highly expressed in the vertebrate lens where cataracts are commonly associated with crystalline protein deficiencies (Liu et al. 2001; Duncan and Zhao 2007). The present study defines a 208-kb genomic interval for the *MCOA* locus by identical-by-descent (IBD) mapping of multiple horse breeds.

Materials and methods

Horse material

Four hundred sixty-five horses were genotyped in this study (Table 1), including 362 Rocky Mountain horses, 57 American Miniature horses, 22 Kentucky Mountain Saddle horses, and 24 Icelandic horses. All Rocky Mountain horses evaluated in this study were derived from four half-sibling families and have been described previously (Ewart et al. 2000). The distributions for phenotypes of Rocky Mountain horses were 72 with MCOA, 222 with Cyst phenotype, and 68 unaffected horses. Distribution of phenotypes for the remaining breeds was as follows: American Miniature Horse: 3 MCOA, 14 Cyst, 40 unaffected; Kentucky

Mountain Saddle Horse: 4 MCOA, 4 Cyst, 14 unaffected; and Icelandic Horse: 4 MCOA, 14 Cyst, 6 unaffected.

Phenotype assessment

Eye examinations were performed as described previously (Ramsey et al. 1999a). Briefly, direct and indirect pupillary light reflexes were first assessed. Following pharmacologic mydriasis with 1% tropicamide administered topically, a complete ophthalmic examination consisting of slit lamp biomicroscopy, indirect ophthalmoscopy, and applanation tonometry was performed. The study was approved by the Michigan State University Institutional Animal Use and Care Ethics Committee for all breeds except the Icelandic Horse; for that breed, the study was approved by the Ethics Committee for Animal Experiments in Uppsala, Sweden.

Microsatellite genotyping

Fourteen microsatellite markers spanning approximately 12 Mb on ECA6q were used for genotyping (Table 2, Supplementary File 1). Seven of these markers were identified previously (*TKY570*, *TKY412*, *TKY284*, *UPP5*, *UPP6*, *UPP7*, and *TKY952*), while seven were novel and developed from the horse genome sequence (EquCab2) (UCSC Genome Browser, http://genome.ucsc.edu/) by displaying tracks from simple tandem repeats recognized by Tandem Repeats Finder (Benson 1999). Initially, genotyping was done on a limited number of horses for a wide interval. Subsequently, an increased number of horses were genotyped for a smaller region that was approximately 2–3 Mb wide. After identifying recombinant horses, further marker genotyping focused on these samples. Table 2 summarizes the number of horses that were genotyped for each marker. Primers for novel microsatellites were designed using Primer3 (Rozen and Skaletsky 2000). PCR reactions were performed as described previously (Andersson et al. 2008). Amplified fragments were multiplexed when possible and separated using a MegaBACE™ 1000 instrument (GE Healthcare Bio-Science Corp., Piscataway, NJ) according to the manufacturer's recommendations. Results were analyzed using Genetic Profiler ver. 2.4 (Amersham Bioscience, GE Healthcare Bio-Science Corp.). Parentage testing was performed according to standard procedures (17 microsatellite markers, Equine Genotypes™ Panel 1.1, Finnzymes Oy, Espoo, Finland) at the Animal Genetics Laboratory, Swedish University of Agricultural Sciences, Uppsala.

SNP genotyping

Six single nucleotide polymorphism (SNP) markers were selected in regions lacking microsatellite markers. Custom TaqMan SNP Genotyping assays [Applied Biosystems (ABI), Foster City, CA] were used for genotyping five of the SNPs. Probe and primer designs were obtained from the ABI web page (http://www5.appliedbiosystems.com/tools/cadt/) using the custom genotyping assays order option. The ABI PRISM 7900 HT sequence detection system for 384-well format (ABI) was used for the analysis. The six selected SNP markers were genotyped in 123–465 horses (Table 2). One additional SNP was investigated in 33 horses by traditional Sanger sequencing (see the next subsection). The *PMEL17* SNP in exon 11 was interrogated using pyrosequencing as described previously (Andersson et al. 2008).

Sanger sequencing of *SMARCC2* and *IKZF4*

Two candidate genes, *SMARCC2* and *IKZF4*, within the identified genomic interval were sequenced using the Sanger method. Three horses with MCOA and two unaffected controls were used to sequence *SMARCC2*, while two MCOA horses and one unaffected control were used for sequencing *IKZF4*. The generated DNA sequences were also compared with the horse reference genome (Wade et al. 2009). Since none of the genes are well annotated in the horse genome, exon number, size, and position were deduced by displaying the track "None-Horse mRNA" from GenBank. Sequencing primers were designed to amplify 500–700-bp fragments using Primer3 (Rozen and Skaletsky 2000). Primer sequences are listed in Supplementary File 1. All exons, as well as some introns, the untranslated regions (UTRs), and certain evolutionary

Table 1 Number of horses investigated in the present study

Breed	MCOA phenotype	Cyst phenotype	Unaffected	Unaffected (Silver)	Total
Rocky Mountain Horse	72	222	59	9	362
Kentucky Saddle Horse	4	4	13	1	22
American Miniature Horse	3	14	37	3	57
Icelandic Horse	4	14	3	3	24
Total	83	254	112	16	465

The unaffected horses that are Silver represent the nonpenetrance horses in this study

Table 2 Genotyping results for 21 markers among horses grouped according to phenotypic status

Position	Marker	Total	MCOA				Cyst				Unaffected			
		n	*n*	*f* (MM)	*f* (Mm)	*f* (mm)	*n*	*f* (MM)	*f* (Mm)	*f* (mm)	*n*	*f* (MM)	*f* (Mm)	*f* (mm)
66793555	*TKY570*	82	26	0.31	0.62	0.08	35	0.11	0.57	0.31	21	0.00	0.43	0.57
70589359	*TKY412*	178	41	0.29	0.44	0.27	105	0.03	0.43	0.54	26	0.00	0.04	0.96
72902566	*MS18*	433	74	0.27	0.39	0.34	231	0.02	0.46	0.52	120	0.01	0.19	0.80
73607795	*MS1*	434	75	0.85	0.13	0.01	231	0.22	0.71	0.08	120	0.03	0.27	0.71
73640494	*N1*	33	18	0.78	0.22	0.00	3	0.00	1.00	0.00	12	0.83	0.17	0.00
73658168	***MS14***	**428**	**79**	**0.97**	**0.03**	**0.00**	**230**	**0.45**	**0.55**	**0.00**	**114**	**0.26**	**0.40**	**0.33**
73665305	***Pmel17-ex11***	**462**	**83**	**0.98**	**0.02**	**0.00**	**245**	**0.00**	**1.00**	**0.00**	**125**	**0.00**	**0.13**	**0.87**
73722621	***MS3***	**453**	**83**	**0.98**	**0.02**	**0.00**	**238**	**0.08**	**0.92**	**0.00**	**124**	**0.02**	**0.25**	**0.73**
73726092	***8345***	**135**	**34**	**0.97**	**0.03**	**0.00**	**30**	**0.43**	**0.57**	**0.00**	**52**	**0.37**	**0.48**	**0.15**
73768488	***TKY284***	**365**	**64**	**0.97**	**0.03**	**0.00**	**189**	**0.06**	**0.94**	**0.00**	**105**	**0.00**	**0.15**	**0.85**
73788011	***8377***	**129**	**32**	**0.97**	**0.03**	**0.00**	**40**	**0.70**	**0.30**	**0.00**	**50**	**0.64**	**0.32**	**0.04**
73835084	***MS13***	**447**	**77**	**0.97**	**0.03**	**0.00**	**234**	**0.03**	**0.97**	**0.00**	**124**	**0.01**	**0.15**	**0.85**
73904952	*8453*	123	31	0.65	0.29	0.06	39	0.03	0.56	0.41	50	0.10	0.56	0.34
73968182	*8475*	130	33	0.67	0.27	0.06	45	0.02	0.60	0.38	49	0.00	0.24	0.76
74029118	*MS21*	392	71	0.82	0.14	0.04	198	0.37	0.57	0.06	115	0.19	0.51	0.30
74063248	*4963*	123	32	0.66	0.28	0.06	34	0.03	0.56	0.41	50	0.10	0.56	0.34
74667009	*UPP6*	422	71	0.86	0.14	0.00	228	0.02	0.91	0.07	115	0.00	0.10	0.90
75475234	*UPP7*	340	69	0.93	0.07	0.00	209	0.24	0.75	0.01	54	0.15	0.43	0.43
76228564	*UPP8*	184	46	0.85	0.15	0.00	101	0.06	0.89	0.05	37	0.00	0.16	0.84
78856446	*MS4*	91	32	0.66	0.31	0.03	36	0.50	0.44	0.06	23	0.61	0.35	0.04
79472875	*TKY952*	83	26	0.58	0.42	0.00	36	0.11	0.61	0.28	21	0.05	0.29	0.67

The 264-kb associated haplotype is marked in bold. The disease allele is depicted as M and an alternative allele as m. Sixteen nonpenetrance horses are included as unaffected individuals

conserved elements were sequenced (Supplementary File 1). Sequencing reactions were carried out as described previously (Brunberg et al. 2006) and results were analyzed using CodonCode software (CodonCode Aligner ver. 1.6.3, CodonCode Corporation, Dedham, MA). Consensus sequences were deposited in GenBank (accession Nos. HQ331540 and HQ331541 for *SMARCC2* and *IKZF4*, respectively).

Results

Haplotype analysis

Genotyping of 14 microsatellite and 7 SNP markers firmly identified a 264-kb genomic interval for the *MCOA* locus (Tables 2, 3 haplotype A, B, C1, and D1). SNP markers *N1* and *8453* represented the 5′ and 3′ borders of the interval, respectively. Four of the microsatellite markers (*MS14*, *MS3*, *TKY284*, and *MS13*) and three SNPs (*PMEL17ex11*, *8345*, and *8377*) reside within the interval. Table 2 displays the results from the genotyping of each marker. With the exception of markers *PMEL17* and *TKY284*, disease-associated alleles are also common in unaffected horses. Phased data of chromosomes that carry the mutation is depicted in Table 3. Forty-eight percent of the individual genotypes were inferred based on surrounding marker information. This was performed only when the haplotype could be assessed with certainty. In total, a combination of 457 affected and unaffected horses of four breeds were used to define the interval (see schematic figure in Supplementary File 2). This corresponds to 409 analyzed chromosomes that carry the MCOA mutation (including horses with both the MCOA and the Cyst phenotype). Of 83 MCOA horses included in this study, 81 were homozygous for the disease-associated haplotype; the remaining two horses were diagnosed clinically with MCOA but carried only one copy of the disease haplotype. Of the 254 horses with the Cyst phenotype, 245 were heterozygous for the disease-associated haplotype and one horse had two copies of the disease haplotype. The remaining eight horses had individually unique haplotypes (see Supplementary File 2). These horses were parentage-tested to determine whether DNA and/or blood sample error had occurred. However, parentage results were accurate. Our genetic data also revealed 16 horses in which the Cyst genotype showed nonpenetrance. These horses represent only 6% of all genetically defined heterozygous horses.

Table 3 Phased data of 13 markers

Haplotype	No. of Chr	*MS1*	*N1*	*MS14*	*PMEL17-ex11*	*MS3*	*8345*	*TKY284*	*8377*	*MS13*	*SMARCC-int24*	*SMARCC-int19*	*8453*	*8475*	Breed
A	355	269	T	*247*	*T*	*233*	*C*	*177/175*	*C*	*222*	*G*	*G*	A	T	RH, KY
B	12	263	G	*247*	*T*	*233*	*C*	*177*	*C*	*222*	*G*	*G*	G	A	RH, KY
C.1	10	263	G	*247*	*T*	*233*	*C*	*177*	*C*	*222*	*G*	*G*	A	A	MINI
D.1	21	263	G	*247*	*T*	*231*	*C*	*177*	*C*	*222*	*G*	*G*	G	A	IS
D.2	1	263	G	**247**	**T**	**231**	**C**	**177**	**C**	**222**	G	T	G	A	IS
C.2	10	263	G	**247**	**T**	**233**	**C**	**177**	**C**	**222**	A	T	G	A	MINI
Position[a]		0	33	50	58	115	118	161	180	227	240	242	297	360	

Six haplotypes were identified in the four analyzed breeds. The number of chromosomes (Chr) and the respective breed are listed for each haplotype. The 264-kb associated haplotype is marked in italics, while the 208-kb haplotype that includes the horses recombinant at the *SMARCC2* locus is marked in bold

[a] The chromosome position of marker *MS1* is 73607795 and is here set as the reference point zero (0). The distance to the following markers are given in kilobases

Sanger sequencing of *IKZF4* and *SMARCC2*

In total, 13.6 kb of *IKZF4* (87%) were evaluated for polymorphisms, including all exons and conserved sequences within introns and 3.6 kb upstream of the gene (Supplementary File 1). Comparison among MCOA horses and unaffected control horses revealed three intronic polymorphic sites (Supplementary File 3). Two of these were SNPs (introns 3 and 4) and not completely associated with the disease. The third polymorphism was a homozygous adenine insertion (intron 5) in both MCOA horses that was not present in either of the unaffected controls or the reference genome.

Resequencing of the *SMARCC2* gene covered 12.6 kb (76%) and included all 29 exons (Supplementary File 1) and intronic conserved elements. Ten SNPs were detected and two of them were positioned within coding regions (Supplementary File 3). Only two SNPs, in intron 19 and 24, respectively, matched the correct mode of inheritance in the horses selected for resequencing. Since *SMARCC2* is positioned close to the 3′ downstream border of the 264-kb IBD interval, the intron 19 SNP was genotyped in recombinant horses. These are the horses with the shortest IBD region compared with the majority of horses (Table 3, haplotypes B-D). The disease-associated allele for this SNP is G, but genotyping revealed nine genetically well-defined Cyst horses as being TT (Table 3, haplotypes C2 and D2). Two additional American Miniature horses displaying the Cyst phenotype can be phased as either C1 or C2 but have been labeled as C2 due to a close relatedness to the other horses carrying the C2 haplotype. Furthermore, a subset of horses, including the newly identified recombinants, was also genotyped for the SNP in intron 24. *SMARCC2* is positioned on the minus strand and hence intron 24 is upstream of intron 19 according to the depicted interval. The disease-associated allele for this SNP is also guanine but the C2 haplotype holds an adenine at this position. These horses thus shortened the genetic interval by 57 kb, providing a minimum shared haplotype of 208 kb.

Discussion

Identical-by-descent mapping was used to firmly define a 208-kb genomic interval on horse chromosome 6 for the *MCOA* locus. This has shortened the previous 4.9-Mb MCOA genetic interval by a factor of 20, which has unlinked 176 other genes from the disease. In addition, a large number of horses were utilized to verify that the mutation causing MCOA syndrome does have a clear additive effect and that the two distinct phenotypic categories, Cyst and MCOA, are caused by one and two copies of the mutant allele, respectively ($p = 2.2 \times 10^{-16}$). Results of the study reported here are based on analysis of 21 genetic markers in 465 horses from four different breeds. The identified interval is a gene-dense area of the genome and includes 15 genes (see Supplementary File 4). Based on information on gene function from studies in other species, two candidate genes within the interval, *SMARCC2* and *IKZF4*, were evaluated for DNA polymorphisms using Sanger sequencing. Two intronic SNPs that segregated with the disease phenotype were detected in *SMARCC2*. After analysis of these SNPs in recombinant horses, the interval for the MCOA syndrome was shortened by 57 kb (from 265 to 208 kb). Sequencing also revealed one disease-associated insertion in intron 5 of *IKZF4*. However, since this polymorphism resides within a non-coding region, it is less likely than the *PMEL17* mutation to have a phenotypic consequence. Nevertheless, this variant will be tested in a larger number of horses.

Another candidate gene within this interval is *PMEL17*. A missense mutation in this gene is most likely causative of the Silver coat color and this mutation may have pleiotropic effects. The protein PMEL17 is involved in the production of eumelanin, but its precise function remains unknown (Theos et al. 2005). Mutations within this gene are associated with dilution of eumelanin in several species, including mouse, chicken, zebrafish, dog, cow, and horse (Kwon et al. 1995; Kerje et al. 2004; Schonthaler et al. 2005; Clark et al. 2006; Brunberg et al. 2006; Kuehn and Weikard 2007). The different species vary in the type and the location of the mutation they exhibit, although several mutations reside near the C-terminal portion of the protein.

To date, ocular defects associated with known *PMEL17* mutants have been reported in zebrafish and dog (Schonthaler et al. 2005; Clark et al. 2006), which supports *PMEL17* as a candidate gene for MCOA syndrome in horses. Conversely, eye defects in these species appear dissimilar, although they are not so easily compared. In zebrafish *fdv* mutant larvae, the retinal pigment epithelium (RPE) melanosome biogenesis is impaired, leading to photoreceptors with fewer, shorter, and misaligned outer segments. The retina is partially recovered in the adult *fdv* zebrafish. The zebrafish RPE melanosomes have an aberrant shape and contain less melanin, giving the eye and the body a lighter appearance. Merle dogs that are homozygous for the proposed *SILV* mutation exhibit several different aberrant eye phenotypes such as starburst/irregular dropped pupils, heterochromia, and microphthalmia. Like the zebrafish, affected dogs have defects in the pigmentation of skin, coat, and RPE.

Recently, chickens homozygous for the *Dominant white* mutation, a 6-bp insertion (ins723–725) within the transmembrane region of *PMEL17*, were found to be free from ocular abnormalities and visual impairment (Karlsson et al. 2009). *Dominant white* mutant chickens appeared less pigmented than wild-type chickens in the outer layers of the retina, but the RPE seemed to be unaltered. Further investigations of eyes of chickens that carry the *Dun* allele would be helpful since they have the exact same missense mutation (R740C) as the horse and also have a 12-bp deletion (del731–735).

The degree of ocular pigmentation and MCOA are poorly correlated. All but eight horses, diagnosed with MCOA syndrome, carried the *Silver* mutation, however their RPE appears to be unaltered (Ramsey et al. 1999a; B. Ekesten, unpublished). Pigmented uveal tissues (iris, ciliary body, choroid) may also be darkly pigmented or lightly pigmented in both MCOA-affected and normal horses. Nevertheless, the fact that the MCOA syndrome has been diagnosed in all breeds where the Silver coat color is present favors *PMEL17* as a candidate gene.

Several other possible candidate genes exist in the interval evaluated. According to AceView, a database that gathers all cDNA sequences available in public databases, 14 of 15 genes in the associated interval are variably expressed in ocular tissues (Thierry-Mieg and Thierry-Mieg 2006). A similar search in the Mouse Gene Expression Database (http://www.informatics.jax.org/expression.shtml) showed that six of the genes were known to be expressed in ocular tissues. To date, the remaining genes were either not found (six genes) or there was no evidence of expression (three genes). In addition to *PMEL17*, *IKZF4*, and *SMARCC2*, a literature search identified four other genes within the interval, *SUOX*, *CDK2*, *ErbB3*, and *RAB5B*, that have documented connection to eye development or function. Mutations in *SUOX* can cause *Isolated sulfite oxidase deficiency*, a severe autosomal recessive neurological disorder. One symptom of this disease is dislocation of the ocular lenses (Johnson et al. 2002). The role of the cell cycle protein CDK2 has been studied extensively during cell differentiation and proliferation in the lens (Wang et al. 2005). *ErbB3* is a member of the epidermal growth factor receptor family and activates a pathway leading to cell proliferation and differentiation. Expression of ErbB3 in conjunctiva has been shown to be higher in patients suffering from keratoconjunctivitis sicca (dry eye syndrome) compared with normal subjects (Liu et al. 2000). Although direct evidence for a role of *RAB5B* in ocular development was not established, several other Rab and Rab-associated proteins are known to be involved. Mutations in some of these genes are responsible for the heritable eye defects choroideremia, Warburg Micro syndrome, and Martsolf syndrome (Corbeel and Freson 2008).

In the present study, 16 horses had clinically normal eyes but were heterozygous for the disease haplotype (including the *PMEL17* mutation). Twelve of these had the Silver coat color and four were chestnuts. This nonpenetrance could be explained by genetic background or, more likely, that very small cysts located on the posterior iris or peripheral ciliary body may not have been detected. Conversely, eight horses that had the Cyst phenotype but not the disease-associated haplotype were also identified. However, it is conceivable that these cysts may be attributable to postinflammatory ocular disease or other nonhereditary causes.

Results of this study are of great importance for veterinarians and horse breeders since genetic markers within our identified interval (e.g., *PMEL17* mutation) can be used for genetic testing of horses. Genetic information could be used for diagnosis of MCOA and to advise horse breeders regarding selection of horses for breeding. Horses that are homozygous for the disease-causing alleles exhibit the most severe clinical signs. Therefore, breeding an affected horse (MCOA or Cyst phenotype) with another affected

horse (MCOA or Cyst phenotype) should be avoided as this could produce a horse with MCOA. Genetic testing for *PMEL17* is of particular use since the Silver coat color can be confused with similar coat colors and because it is not possible to detect the mutation on coat colors that do not express any black pigment or have coat color genes such as Gray that could mask the Silver phenotype. So far it remains speculative whether the *PMEL17* mutation causes both the Silver coat color and MCOA syndrome. However, linkage disequilibrium between the two is complete; if the phenotypes are regulated by separate mutations, they are a maximum of 183 kb apart. Because of this, it may be practically impossible to find horses that have recombined between these loci (i.e., carrying a haplotype with the *Silver* mutation but devoid of the mutation causing MCOA syndrome). On both sides of the MCOA interval, it is less than 20 kb between the marker defining the interval border and the markers within the IBD region (18 and 13 kb, respectively). Thus, additional fine mapping with our present horse material will not substantially change the size of the interval. Therefore, future aims include sequencing the entire genetic interval by next generation, deep sequencing to identify all candidate mutations in conserved sequences, and subsequently permitting functional assay evaluations.

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