Pleiotropic effects of pigmentation genes in horses

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Summary

Horses are valued for the beauty and variety of colouration and coat patterning. To date, eleven different genes have been characterized that contribute to the variation observed in the horse. Unfortunately, mutations involving pigmentation often lead to deleterious effects in other systems, some of which have been described in the horse. This review focuses on six such pleiotropic effects or associations with pigmentation genes. These include neurological defects (lethal white foal syndrome and lavender foal syndrome), hearing defects, eye disorders (congenital stationary night blindness and multiple congenital ocular anomalies), as well as horse-specific melanoma. The pigmentation phenotype, disorder phenotype, mode of inheritance, genetic or genomic methods utilized to identify the genes involved and, if known, the causative mutations, molecular interactions and other susceptibility loci are discussed. As our understanding of pigmentation in the horse increases, through the use of novel genomic tools, we are likely to unravel yet unknown pleiotropic effects and determine additional interactions between previously discovered loci.

Coat colour and spotting patterns have long fascinated animal breeders and geneticists alike. The pigmentation genes of mice were one of the first genetic systems to be elucidated and over 130 loci and 1000 mutations have been characterized (**Steingrímsson*et al.* 2006**). Pigmentation was one of the first systems to be studied, originally, because of the ease with which the traits could be identified as well as the variability in coat colour resulting from selective breeding by ‘mouse fanciers’ in the 19th century.

Similarly to the mouse fanciers of the 19th century, breeders selectively breed horses for their beauty and variety of coat colour and patterning (among other traits). While it has been suggested that a particular coat colour might be essential for survival and or have a selective advantage under certain environmental conditions (such as white horses being less attractive to the horse-fly), it is believed that most of the variation observed in breeds of horses today is a result of domestication and selective breeding (**Ludwig *et al.* 2009**; **Horváth *et al.* 2010**).

While the horse falls short of the 130 loci discovered in mice, currently as many as 31 loci have been postulated to be involved in coat colour variation and patterning in domestic horse breeds (**Sponenberg 2009**). With the utility of the equine genome sequence, many more are likely to be discovered in the near future (**Wade *et al.* 2009**). Seventeen different coat colour phenotypes have been investigated at the molecular level, and causative mutations have been identified and/or associations have been reported for eleven different genes (Table 1). Variation in horse coat colour is most often because of mutations that affect functioning of mature melanocytes. The base coat colour of the horse (black, bay, chestnut or seal brown) is determined by two loci (*MC1R*and (*ASIP*) that control the switch between eumelanin and phaeomelanin production (**Marklund *et al.*1996**; **Rieder*et al.* 2001**; **Sponenberg 2009**). Mutations in four additional genes cause dilutions in coat colouring, resulting in cream (*SLC45A2*), silver (*SILV*), champagne (*SLC36A1*), and pearl (*SLC45A2)* dilutes as well as lavender foal (*MYO5A*)
(**Mariat *et al.* 2003**; **Brunberg*et al.* 2006**; **Cook *et al.* 2008**; **Sponenberg 2009**; **Brooks*et al.* 2010***)***. Patterning has been shown to be controlled by genes involved in the migration, proliferation and survival of melanocyte precursor cells, such as *KIT*(sabino-1, tobiano and dominant white) and *endothelian receptor type B*gene (*EDNRB*) (frame overo), and is also caused by mutations affecting melanocyte stem cell populations such as in the *STX17* gene (grey)
(**Metallinos*et al.* 1998**; **Santschi *et al.*1998**; **Yang *et al.* 1998**; **Brooks & Bailey 2005**; **Brooks *et al.*2007**; **Haase *et al.* 2007**; **Pielberg *et al.* 2008**). Other dilutions and patterning traits have been mapped, but the causative mutations remain unknown; these include dun, roan, leopard complex, and white face and leg markings (Table 1, Reviewed in **Rieder 2009**).

Table 1. Coat colour genes characterized in the horse.

Coat colour phenotype	Genes
Chestnut	*MC1R*

Coat colour phenotype	Genes
Bay	*MC1R* and *ASIP*
Black	*MC1R* and *ASIP*
Seal brown	*MC1R* and *ASIP*
Cream	*SLC45A2*
Pearl	*SLC45A2*
Silver	*SILV*
Champagne	*SLC36A1*
Lavender foal	*MYO5A*
Sabino-1	*KIT*
Tobiano	*KIT*
Dominant white	*KIT*
Frame overo	*EDNRB*
Grey	*STX17*
Leopard complex	Associated with *TRPM1*
Roan	Associated with *KIT*
White face and leg markings	Associated with *KIT*, *MC1R*, and *MITF*

Melanocytes are derived from embryonic cells of neural crest origin. Neural crest-derived melanocytes are found in the skin, hair, certain layers of the eye (uveal melanocytes), the inner ear and leptomeninges. In addition, embryonic stem cells of neural crest origin also give rise to bone, cartilage, adipose, endocrine cells and several types of neurons and glia (**Le Douarin & Kalcheim 1999**). Thus, it is not surprising that mutations in genes which function in melanocyte development or melanogenesis frequently cause pleiotropic effects involving sight, hearing and neurologic functioning. Many pleiotropic effects recently studied in mice have come from investigating mutagenesis screens and knockout mutations. However, the broad variation in colour and pattering in and among horse breeds that has occurred as a result of domestication makes the horse an excellent model to unravel yet unknown causes of disease that are associated with pigmentation, several of which are currently being investigated. The coat colour mutations that have been reported to date were recently reviewed by **Rieder (2009)**. Thus, this review focuses only on those pigmentation genes investigated in the

horse, which either have known pleiotropic effects or have been associated with a disorder but are still under investigation.

Pleiotropic pigmentation genes

Lethal white foal syndrome (LWFS) and the *endothelin receptor type B gene (EDNRB*)

Lethal white foal syndrome was the first pigmentation-related pleiotropic effect to be identified at the molecular level in the horse. Lethal white foal syndrome has been associated with the frame overo pattern (Fig. 1a) (**McCabe *et al.* 1990**). The term 'frame' comes from the phenotypic appearance of this spotting pattern in which the pigmentation 'frames' the horse. Therefore, the white spotting usually occurs in the middle of the sides of the flank, and neck as well as ventrally, but rarely dorsally, unless other pigmentation gene mutations are involved. Variation in the amount of white spotting does occur and can complicate proper classification. Frame horses often have partially depigmented irides involving either one or both eyes (blue or partially blue eyes). However, to date, no eye disorders have been associated with this phenotype.

Figure 1.

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Pigmentation phenotypes in the horse associated with pleiotropic effects. (a) Frame overo, which in the homozygous condition causes lethal white foal syndrome. (b) Lethal white foal resulting from a homozygous mutation in *EDNRB*.(c) Splash white is often associated with deafness; however, the genetic mutation has not been determined. (d) Lavender foal caused by a homozygous mutation in *MYO5A*.(e) Flea bitten grey. (f) Dapple grey. (g) Grey horse with melanoma in atypical area. (h) 'Leopard' pattern, which is heterozygous for *LP* and unaffected for congenital stationary night blindness (CSNB). (i) 'Few spot' pattern, which is homozygous for *LP* and been associated with CSNB, and is likely caused by a mutation in *TRPM1*. (j) Silver dapple caused by a mutation in *PMEL17*and associated with multiple congenital ocular anomalies.

The frame pattern is inherited as a dominant allele (**Bowling 1994**). As such, all frame horses are heterozygous for the mutation. However, the result of the homozygous condition is LWFS. This syndrome is characterized by foals born with a complete or near-complete white coat (Fig. 1b). These horses are also affected with intestinal aganglionosis, which is the absence of enteric ganglion cells (**Hultgren 1982**and **McCabe *et al.* 1990**). Because of the lack of proper enervation of the intestine, foals develop intestinal obstruction within 24 h of birth, as food cannot be passed through the lower digestive tract. This obstruction quickly results in death. Three research groups independently identified the mutation responsible

for this disease using a candidate gene approach (Metallinos *et al.* 1998; Santschi *et al.* 1998; and Yang *et al.* 1998). Mutations in the endothelin signalling pathways, involving both *EDNRB* and *endothelin 3 (EDN3)*, cause similar phenotypes in humans (Hirschsprung disease) and in *Ednrb* and *Edn3* knockout (KO) mice. In addition to aganlionosis, humans homozygous (but not heterozygous) for a mutation in *EDNRB* have hypopigmentation, and KO mice for *EDNRB* but not *EDN3*are almost completely white (Hosoda *et al.* 1994; Puffenberger *et al.* 1994). Thus, *EDNRB* was chosen as the candidate gene to sequence in the horse. A dinucleotide missense mutation in the first exon of this gene was identified as the cause (NM_001081837.1:c.353_354delinsAG, resulting in an amino acid substitution of lysine for isoleucine (p.Ile118Lys) (Metallinos *et al.* 1998; Santschi *et al.* 1998; and Yang*et al.* 1998) (Table 2). The substitution to a charged amino acid is predicted to impact on the function of the first transmembrane domain of this seven transmembrane G-coupled receptor protein. The exact mechanism of how this substitution affects function, either in receptor localization or in signalling, remains to be determined.

Table 2. An overview of the genes involved in pigmentation and their pleiotropic effects studied in the horse.

Coat colour or pattern	Gene	Locus	DNA mutation	Predicted function of mutation	Pleiotropic effects observed	Gene function
Frame overo pattern	*EDNRB*	ECA17	NM_001081837.1:c.353_354delinsAG	Amino acid substitution (p.Ile118Lys) disrupting function	Lethal white foal syndrome in homozygotes (absence of enteric ganglion cells); deafness susceptibility	Known to affect proliferation, migration, and differentiation of neural crest-derived melanocytes and enteric ganglia cells.
Splash white pattern	Unk	Unk	Unk	Unk	Deafness susceptibility	Unk
Lavender coat dilution	*MYO5A*	ECA1	ECA1g. 138235715del	Frame shift resulting in a truncated protein	Homozygotes have neurological symptoms leading to death	Proposed to disrupt trafficking of melanosome in melanocytes and synapse molecules in neurons.
Grey pattern	*STX17*	ECA25	ECA25g.6575277_6579862dup	Cis-acting regulatory element that upregulates both STX17 and NR4A3	Melanoma in which homozygotes are more susceptible	Proposed to promote melanocyte proliferation, causing melanoma in the dermis and depletion of stem cell melanocyte populations associated with hair follicles.
Leopard complex pattern	*TRPM1*	ECA1	Unk	Downregulate TRPM1	Homozygotes are affected with congenital stationary	Shown to be the selective cation transduction channel in the ON bipolar cell

Unk, unknown.

Coat colour or pattern	Gene	Locus	DNA mutation	Predicted function of mutation	Pleiotropic effects observed	Gene function
					night blindness	pathway for vision in low light conditions. Also proposed to play a role in the storage of melanin and potentially in melanocyte migration.
Silver dilution	*SILV*	ECA6	DQ665301:g.1457C>T	Amino acid substitution (p.Arg618Cys) disrupting function	Association of silver with multiple congenital ocular anomalies	Shown to be involved in biogenesis of premelanosome and perhaps specifically the eumelanosome. Work in dogs suggests functional role in eye development

Similarly to melanocytes, enteric ganglia cells are derived from the neural crest and thus endothelin signalling is important for the proliferation, migration and differentiation of both cell populations. While both melanocytes and enteric ganglia are derived from neural crest precursors, they migrate along two different pathways; cells destined to become enteric ganglia migrate along a dorso-ventral pathway while those destined to become melanocytes migrate along a dorso-lateral pathway (Pla & Larue 2003). Thus, the timing and expression of endothelin signalling appears to have several effects on these two cell types. EDN3/EDNRB signalling represses differentiation of early-stage enteric neural crest cells, thus allowing for migration of these cells and sensitivity to mitogenic effects by other molecules (Pla & Larue 2003). Later in development, EDNRB-mediated signalling is required after the establishment of enteric neuroblasts for correct innervations (McCallion & Chakravarti 2001). Similarly, in melanocytes, endothelin signalling plays a role in migration and terminal differentiation; however, dorso-lateral migration appears to begin later (Reid *et al.* 1996; Pla & Larue 2003). While the precise intracellular signalling pathways for melanocytes that are activated by EDN3 binding to EDNRB are not known, signalling through Gq coupling of EDNRB, involving diacyglycerol, inositol triphoshpate and calcium messengers, has been shown to be involved in enteric glia development (Imamura *et al.*2000). Determining the intracellular signalling cascade for melanocytes in both humans and horse would help resolve whether signalling through EDNRB is different in the two tissue types. Furthermore, if different intracellular signalling pathways are observed for horse and human, this could explain why, in the heterozygous condition, mutations in EDNRB cause white spotting phenotypes in horses but not in humans (Metallinos *et al.* 1998; McCallion & Chakravarti 2001).

As mentioned previously, variability in the amount of white exists for frame horses. Furthermore, in a study conducted by Santschi *et al.*2001, it was shown that the association with p.Ile118Lys and the frame phenotype was not 100%; non-frame horses had the mutation, and horses with a phenotype of frame had a genotype of p.118Ile (wild type). Thus, it is likely that this mutation is not fully penetrant in the heterozygous condition and can be masked by modifier genes (Santschi *et al.* 2001). Additionally, it is also likely that mutations in other genes may cause a similar frame phenotype. Based on work in mice and humans, a sex effect and the tyrosine kinase receptor *KIT*, its ligand *KITLG*and transcription factors *SOX10* and *PAX3* may be important modifier genes for further exploration (McCallion & Chakravarti 2001). Ten per cent of the tobiano horses (a white spotting pattern different from frame overo in which the white crosses the top line) tested were heterozygous for the p.Ile118Lys mutation, but did not display a recognizable overo pattern (Santschi *et al.*2001). This strongly suggests that *KIT* is likely a modifier gene, and this interaction should be explored in the horse.

Deafness and the *endothelin receptor type B gene (EDNRB*)

Humans homozygous for a mutation in *EDNRB* with a specific form of Hirschsprung disease, known as Shah–Wardenburg syndrome (WS4), also have sensorineural deafness and bicoloured irides, in addition to aganglionosis and hypopigmentation (Puffenberger *et al.* 1994). A 318 bp deletion in *EDNRB* was developed as a mouse model for WS4. This mouse also had pigmentation anomalies, deafness, and megacolon (Matsushima *et al.* 2002). The neurosensory deafness associated with WS4 in the mouse model and humans is believed to be caused by the lack of melanocytes in the inner ear. These melanocytes are thought to be required for the formation and integrity of the stria vascularis and

maintenance of the endocochlear potential (Pla & Larue 2003). White spotting has also been associated with deafness in dogs and cats, but *EDNRB* has not yet been associated (Geigy *et al.* 2007; Strain *et al.* 2009).
Prior to the identification of the mutation for LWFS, deafness was anecdotally associated with this disorder. However, it was not known whether all lethal white foals were affected (McCabe *et al.* 1990). Recently, a study by Magdesian and colleagues investigated deafness in American Paint Horses, including those with the NM_001081837.1:c.353_354delinsAG mutation in *EDNRB*(Magdesian *et al.* 2009). Among those horses that were confirmed deaf by brainstem auditory-evoked responses, and suspected deaf by their owners, most had the coat patterning of either splashed white (causative gene mutation not yet known, Fig. 1c, Table 2) or frame-splashed white blend. Three lethal white foals were confirmed deaf prior to death. Furthermore, 91% of the horses confirmed or suspected to be deaf had the NM_001081837.1:c. 353_354delinsAG mutation (Magdesian *et al.* 2009). This association suggests that *EDNRB* may play a role in deafness; however, similar to the frame coat patterning, other genes are likely to be involved. One of these is probably the gene and/or genes responsible for splash white. Splash white has not been molecularly characterized in the horse. Unravelling the genetic mechanism of splash will likely help elucidate the interaction of splash and frame patterning and the association of deafness with these phenotypes. Additionally, cases of WS4 in humans have been shown to also be caused by mutations in *EDN3* and *SOX10*; thus, these candidate genes should be investigated for their association with deafness in the horse (Price & Fisher 2001). Furthermore, while all of the lethal white foals in Magdesian study were deaf, the sample size was small ($N = 3$). Thus, it cannot be concluded that this is the case for all lethal white foals.

Lavender foal syndrome (LFS) and the *myosin VA*gene (*MYO5A*)

The most recently elucidated pleiotropic effect involves LFS, also known as coat colour dilution lethal. Lavender foal syndrome is inherited as a lethal recessive disorder in the Arabian breed, which is primarily of Egyptian descent. This disease was first described by Bowling as light-coloured foals that failed to nurse and died shortly after birth (Bowling 1996). The coats of these animals are often described as a lavender-like colour, and thus the disorder was named for this unique characteristic (Fig. 1d). However, the coat can range from pale grey to light chestnut. The syndrome is characterized by several neurological signs involving tetany (involuntary muscle contractions), opisthotonus (hyperextension of the head and neck), nystagmus (involuntary eye movements), and paddling leg movement (Bowling 1996; Fanelli 2005).
The genetic mutation causing this disorder has very recently been discovered by an SNP-based whole-genome association approach (Brooks*et al.* 2010). The trait mapped to a 10.5 -Mb region on ECA1 containing the candidate genes *myosin VA* (*MYO5A)* and *ras-associated protein RAB27A*(*RAB27A)*, which cause similar disorders in mice and humans (Griscelli syndrome). In addition to neurological abnormalities, mutations in *RAB27A* often cause immunological disruptions. Because such disruptions are not detected in LFS, *MYO5A*was thus chosen as the candidate gene to investigate further. A single-base deletion in exon 30 (ECA1 g.138235715del) is suspected to cause a frame shift leading to a premature stop codon in a highly conserved region of the gene (Brooks *et al.* 2010) (Table 2). Thus, the authors have proposed that this prematurely terminated protein would not be able to effectively bind cargo for intracellular transport. In the case of melanocytes, unlike those mutations that disrupt melanocyte migration and differentiation (such as *EDNRB*described above), this deletion would alter the function of a mature melanocyte, in that melanosome trafficking to the periphery of the cell for transfer to the keratinocyte would be disrupted (Marks & Seabra 2001; Brooks *et al.*2010). Likewise, in nerve cells, glutamate receptors and secretory granules would not be transported properly, and thus this could explain the various neurological defects observed (Goda 2008; Brooks*et al.* 2010). Additional work is needed to confirm the underlying biochemical mechanisms. Furthermore, while a dilution in pigmentation has not been observed in heterozygotes with this deletion (Samantha Brooks personal communication), there is speculation that LFS carriers may have a mild survivable epileptic condition (Fanelli 2005). Future studies will likely investigate pigment density and epileptic condition in heterozygotes.

Melanomas and the *syntaxin 17* gene (*STX17*)

Previous studies have reported that approximately 80% of horses with the grey coat pattern will develop melanomas (Valentine 1995; Sutton & Coleman 1997; Johnson 1998). Grey is a progressive coat colour phenotype that occurs in many breeds of horses, and in some breeds, such as Lipizzaner and Andalusian, it is the predominant colour. Grey horses progressively acquire white hairs throughout the coat as they age, and the greying process (rate and location) varies from horse to horse. Some horses will grey first in the mane and tail hairs while others will lose pigment last in the mane and tail (Sponenberg 2009). Additionally, some horses will retain small flecks of pigmented hairs to display a 'flea-bitten' appearance (Fig. 1e), while others will retain pigmented hairs that outline areas of depigmented hairs to display a 'dappled' appearance (Fig. 1f). Furthermore, some grey horses will develop skin depigmentation (similar to vitiligo in humans) in addition to hair depigmentation (Sutton & Coleman 1997). It is speculated that some of the horse to horse variation in the greying process is likely due to modifier genes that have not yet been investigated. 'Grey' is said to be epistatic to all other coat colours and patterns, as horses that inherit the grey duplication will eventually lose pigment as they age, and any underlying coat colour or pattern will be masked by 'grey'.
'Grey' is inherited as dominant gene. The 'grey' condition was mapped to ECA25 by three independent groups (Henner*et al.* 2002; Locke *et al.* 2002; Swinburne *et al.* 2002) (Table 2). The status of the horse genome at that time did not allow for the identification of candidate genes from that region. Therefore, Pielberg and colleagues identified SNPs in genes on ECA25 to refine the map position. They successfully defined a region that corresponds to approximately 6.9 Mb on human chromosome 9q (Pielberg *et al.* 2005). However, again, no obvious candidate genes causing either

pigmentation defects or melanoma susceptibility were detected in this region. The availability of the horse genome and SNPs detected during the genome sequencing effort made the further refinement and candidate gene investigation possible. Eighteen SNPs in the 6.9 -Mb region were used to define a 350 -kb critical interval, and four genes were investigated from this region, none of which had been previously implicated in pigmentation defects or melanoma susceptibility. **Pielberg *et al.* (2008)** identified a 4.6 -kb duplication in intron 6 of *syntaxin 17* (*STX17*) as the cause of grey (ECA25 g.6575277_6579862dup, as is identifiable with GenBank accession numbers EU606026and EU606027) The authors proposed that this duplication is a cis-acting regulator mutation that upregulates both *STX17* and *nuclear receptor subfamily 4*, *group A, member 3*(*NR4A3*), another gene in the critical interval. Upregulation of STX17 and/or NR4A3 is thought to promote melanocyte proliferation, which in dermal melanocytes leads to predisposition to melanoma development and in hair follicle melanocytes leads to hyperproliferation and a depletion of stem cells. This depletion of the stem cell pool causes the hair to grey as new hairs replace those that are lost. While it is still likely that variation in the greying process and incidence of melanomas is determined by unknown modifiers, Pielberg and coauthors showed that some of this variability is because of the incomplete dominant nature of this mutation. Horses homozygous for the duplication greyed at a faster rate, had more skin depigmentation and less speckling in the coat and also had a higher incidence of melanoma (**Pielberg *et al.*2008**).
The melanomas that appear on horses typically occur as black-pigmented nodules in the dermis of hairless skin, usually under the tail, perianal and genital regions, as well as around the lips and eyelids, although they can occur in other areas (Fig. 1g) (**Seltenhammer *et al.* 2004**). Two cases of neoplasms of the vertebral column in grey horses have also been reported (**Schott *et al.* 1990**). Although melanomas in grey horses show less malignancy to those of solid-coloured horses (**Seltenhammer *et al.* 2003**), metastases do occur in grey horses, and the most common sites are in the lymph nodes, liver, spleen, skeletal muscle, lungs, and surrounding or within blood vessels (**MacGillivray *et al.* 2002**). Genetic mechanisms controlling metastasis in the horse has not been characterized. However, melanoma susceptibility is increased in grey horses that also have a loss of function mutation in agouti signaling protein (ASIP) (**Pielberg *et al.*2008**). ASIP is an antagonist of the melanocortin 1 receptor (MCIR) and is involved in the switch from eumelanogenesis to phaeomelanogenesis. This suggests that in the horse, increased MCIR signalling influences melanoma development. This is in contrast to what has been shown in humans, in which loss of function polymorphisms in *MCIR* and not *ASIP* contribute to melanoma susceptibility (**Fargnoli *et al.* 2006**; **Stratigos*et al.* 2006**; **Brudnik *et al.*2009**). Although loss of function mutations in *MC1R*and melanoma susceptibility have not yet been studied in the horse studying these interactions may help to more clearly discern the differences in the biology and metastasis of equine melanoma from that of human. Additionally, other genes in humans have been shown to play a role in melanoma susceptibility. For example, one polymorphism (NP_000266.2:p.Arg419Glu) in OCA2 is associated with an increased risk of developing malignant melanoma (**Fernandez *et al.* 2009**). In the horse, an SNP in the coding region of *OCA2* (DQ454071. 1:c.346A>G) has been identified, but no association with coat colour phenotype has been determined (**Bellone*et al.* 2006a**). Furthermore, this SNP and others in *OCA2* have not yet been investigated in the horse for melanoma susceptibility. Similarly, an amino acid substitution in MYO7A (NP_000251.3:p.Ser1666Cys) was found to be associated with melanoma risk in humans; this gene has not been investigated in the horse as either a cause of a coat colour phenotype or a melanoma risk factor (**Fernandez *et al.* 2009**).

Congenital stationary night blindness (CSNB) and the *transient receptor potential cation channel, subfamilyM, member 1*gene (*TRPM1*)
Congenital stationary night blindness has been associated with homozygosity for *leopard complex spotting* (*LP*, also known as appaloosa spotting) in the Appaloosa breed (**Sandmeyer *et al.* 2007**). Leopard complex spotting is characterized by patterns of white in the coat that tend to be symmetrical and centred over the hips (**Sponenberg*et al.* 2009**). The extent of white patterning varies widely among individuals, and, like the variation already discussed with frame patterning and grey, this variation is in part because of modifier genes (**Miller 1965**; **Sponenberg *et al.* 1990**; S. Archer and R. R. Bellone, unpublished data). The term ‘leopard’ is derived from one of these patterns in which oval spots of pigment are found in the pattern of white extending over most of the body (Fig. 1h). In addition to the patterning in the coat, *LP* is associated with four other pigmentation traits: striped hooves, readily visible non-pigmented sclera around the eye, mottled pigmentation around the anus, genitalia, and muzzle, and *LP*-specific roaning (**Sponenberg *et al.*2009**). Horses that are homozygous for leopard complex spotting (*LP/LP*) tend to have fewer pigmented spots than heterozygotes in the white patterned areas (**Sponenberg *et al.* 1990**; **Lapp & Carr 1998**) (Fig. 1i). In addition to the Appaloosa breed, many other breeds have leopard complex spotting (Knabstrupper, Noriker, Pony of the Americas, American Miniature, British Spotted Pony, and Australian Spotted Pony, among others); however, association of CSNB with homozygosity for *LP* has not yet been documented in these other breeds.
LP is inherited as an incompletely dominant gene that was mapped to a 6-cM region on ECA1 (**Terry *et al.*2004**). The positional and functional candidate *TRPM1*was implicated as the genetic cause (**Bellone *et al.* 2006b, 2008, 2010a,b**) (Table 2). Similarly to STX17 and NR4A, the role of TRPM1 in pigment production has not been elucidated. However, it was shown that TRPM1 is downregulated in highly metastatic melanoma cells, suggesting that this protein plays an important role in maintaining normal melanogenesis (**Duncan *et al.*1998**). The extent of TRPM1 involvement in horse melanoma has not been determined. Most recently, work in humans has demonstrated that TRPM1 expression correlates directly with melanin concentration, suggesting a potential role for TRPM1 in the storage of melanin (**Oancea *et al.* 2009**).

TRPM1 belongs to the Ca^{2+}transient receptor potential superfamily. Ca^{2+} signalling and sensation have obvious roles in both cell migration and signalling. TRPM1 therefore may also play a role in melanocyte migration, but this remains to be determined.

Congenital stationary night blindness is characterized by a congenital and non-progressive scotopic (low light condition) visual deficit and was first characterized in an Appaloosa horse in 1977 (**Witzel *et al.*1977**). Affected animals occasionally manifest a bilateral dorso-medial strabismus (improper eye alignment) and nystagmus (involuntary eye movement) (**Sandmeyer *et al.* 2007**). Congenital stationary night blindness is diagnosed by a 'negative ERG', which is a dark-adapted electroretinography in which the b-wave is absent and there is a depolarizing a-wave (**Witzel *et al.* 1977**). This is similar to the Schubert–Bornshein type of human CSNB (**Schubert & Bornshein 1952**; **Witzel *et al.* 1978**). The 'negative ERG' is indicative of a defect in depolarizing the ON bipolar cells (the next cells involved in night vision after the rod photoreceptors). The synapse between the ON bipolar cell and the photoreceptor, in causing the depolarizing event, involves the binding of glutamate to its receptor, metabotropic glutamate receptor (MGluR6), which couples to the closure of a cation-selective transduction channel (**Nomura *et al.* 1994**; **Nakanishi *et al.* 1998**). Until very recently, the cation channel involved in ON bipolar cell signalling was not determined. Differential gene expression of *TRPM1* in CSNB-affected Appaloosa horses provided evidence that this gene was the cation-selective channel (**Bellone *et al.* 2008**). Work by Shen *et al.* further supports TRPM1 as the cation channel, as TRPM1 knockout mice exhibit a similar 'negative ERG' (**Shen *et al.* 2009**). Furthermore, very recently several different mutations in TRPM1 have been shown to cause CSNB in humans (**Audo*et al.* 2009**; **van Genderen*et al.* 2009**; **Li *et al.* 2009**).

qRT-PCR analyses showed that *TRPM1* mRNA expression is significantly downregulated in both the skin of homozygotes (*LP*/*LP*) (downregulated by about 200-fold) and the retina of CSNB-affected Appaloosas (downregulated by over 1800-fold), whereas four other linked genes had unaltered mRNA expression (**Bellone *et al.*2008**). This suggested that *TRPM1* was involved in both pigmentation and CSNB. The putative coding region of this gene was investigated for mutations that could explain this difference in expression; none were identified (**Bellone*et al.* 2010a**). Thus, to localize the causative mutation, 70 SNPs spanning over 2 Mb encompassing the *TRPM1* gene were utilized, and a single 173 -kb haplotype associated with *LP* and CSNB (ECA1: 108 197 355–108 370 150) was identified. Illumina resequencing of 300 kb surrounding this haplotype identified six candidate SNP variants for further investigation, which is currently underway and is described in this issue (**Bellone *et al.*2010a,b**). None of the causative mutations in *TRPM1*in humans have been associated with pigmentation differences in the skin. Determining the exact mechanism of *LP* and CSNB in the horse may support melanogenesis processes specific to horse.

Multiple congenital ocular anomalies (MCOA) andthe* silver homolog *gene (*SILV*)

The association of MCOA with the silver colouration in the horse was first documented in 1999 by Ramsey and colleagues. These authors noted a high proportion of affected Rocky Mountain horses who possessed a white mane and tail and dark chocolate body colour (**Ramsey*et al.* 1999**). While the precise association of MCOA with the silver phenotype is still not clear, the mutation in *SILV*causing the silver coat colour has since been determined.

The silver colouration occurs frequently in many breeds, including the Rocky Mountain Horse and closely related breeds Kentucky Mountain Saddle Horse, and Mountain Pleasure Horse as well as the Icelandic horse and American Miniature horse, among others. The prevalence of MCOA and its association with silver colouration have been examined in purebred Rocky Mountain horses in the United States as well as purebred and cross-bred Rocky Mountain Horses in Canada (**Ramsey*et al.* 1999**; **Grahn *et al.* 2008**). The association of MCOA with silver in other breeds such as Icelandic horse and the American Miniature has not yet been reported.

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Conclusion

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non-pigmented sclera around the eye, mottled pigmentation around the anus, genitalia, and muzzle, and *LP*-specific roaning (**Sponenberg *et al.*2009**). Horses that are homozygous for leopard complex spotting (*LP/LP*) tend to have fewer pigmented spots than heterozygotes in the white patterned areas (**Sponenberg *et al.* 1990**; **Lapp & Carr 1998**) (Fig. 1i). In addition to the Appaloosa breed, many other breeds have leopard complex spotting (Knabstrupper, Noriker, Pony of the Americas, American Miniature, British Spotted Pony, and Australian Spotted Pony, among others); however, association of CSNB with homozygosity for *LP* has not yet been documented in these other breeds.

LP is inherited as an incompletely dominant gene that was mapped to a 6-cM region on ECA1 (**Terry *et al.*2004**). The positional and functional candidate *TRPM1*was implicated as the genetic cause (**Bellone *et al.* 2006b, 2008, 2010a,b**) (Table 2). Similarly to STX17 and NR4A, the role of TRPM1 in pigment production has not been elucidated. However, it was shown that TRPM1 is downregulated in highly metastatic melanoma cells, suggesting that this protein plays an important role in maintaining normal melanogenesis (**Duncan *et al.*1998**). The extent of TRPM1 involvement in horse melanoma has not been determined. Most recently, work in humans has demonstrated that TRPM1 expression correlates directly with melanin concentration, suggesting a potential role for TRPM1 in the storage of melanin (**Oancea *et al.* 2009**). TRPM1 belongs to the Ca^{2+}transient receptor potential superfamily. Ca^{2+} signalling and sensation have obvious roles in both cell migration and signalling. TRPM1 therefore may also play a role in melanocyte migration, but this remains to be determined.

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Multiple congenital ocular anomalies (MCOA) andthe *silver homolog* gene (*SILV*)

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Ancillary

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