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Two cysteine substitutions in the *MC1R* generate the blue variant of the arctic fox (*Alopex lagopus*) and prevent expression of the white winter coat

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Abstract

We have characterized two mutations in the *MC1R* gene of the blue variant of the arctic fox (*Alopex lagopus*) that both incorporate a novel cysteine residue into the receptor. A family study in farmed arctic foxes verified that the dominant expression of the blue color phenotype cosegregates completely with the allele harboring these two mutations. Additionally to the altered pigment synthesis, the blue fox allele suppresses the seasonal change in coat color found in the native arctic fox. Consequently, these findings suggest that the MC1R/agouti regulatory system is involved in the seasonal changes of coat color found in arctic fox.
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1. Introduction

At high latitudes, mammals have to face seasonal changes in temperature, light, food availability, and colors in their surroundings. According to the species, there are various physiological adaptations to these environmental changes like hibernation, storage of energy via fat deposition and modification in pelage. The suprachiasmatic nucleus (SCN) of the hypothalamus is the principal component of the mammalian biological clock. The pacemaker of the SCN oscillates with near 24 h period, and coordinates a broad spectrum of physiological, endocrine and behavioral circadian rhythms. Consistent with its role in circadian timing, several experiments have provided evidence that SCN is the locus of the brains endogenous calendar, enabling organisms to anticipate seasonal changes (for review see [8]). Mechanisms of how the circadian rhythm of SCN is translated into seasonal variation and how seasonal variation in coat color is functionally regulated are not well understood.

The arctic fox (*Alopex lagopus*) is a circumpolar inhabitant of the Arctic, and occurs naturally in two color morphs, white and blue. In the white morph the production of pigment is dramatically changed with season. The summer pelage is characterized by a dark dorsal and a light ventral pattern, while the white winter coat is produced as an adaptation to snow and icy environments. A seasonal pelage shift occurs with molting of the highly insulative white winter fur in May to a shorter brown/grey summer fur. The initial growth of the winter fur occurs in September, and continues until early December. The winter fur is 200% thicker than the summer fur [21]. The exact mechanism of the down regulation in

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Fig. 1. Illustrations show the blue variant of the arctic fox in (A) and the winter white variant in (B). Both pictures show the summer coat of a puppy. In (C), an adult blue fox is seen to the left, whereas the winter white variant is seen to the right. Illustrations are of individuals that were included in the study (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

pigment synthesis connected with the change to winter fur is not known.

Blue fox is a color variant of arctic fox, showing a uniform dark grey/blue coat color during the summer. The dominant blue coat color is inherited like a simple Mendelian trait caused by a single gene. In the winter season, the blue fox develops a coat color that is lighter than the summer coat but far more colored compared to the winter white native arctic fox (Fig. 1). At Svalbard, as in several other natural habitats of the arctic fox, 3–5% of the population is of the blue variant.

Dominant acting mutations in the *MC1R* causing a general eumelanizing effect are well known from several mammalian species including mouse, cattle, red fox and sheep. These have been functionally tested in cell assays, and have been found to produce a constitutively active receptor causing eumelaninization mediated through the cAMP pathway [11,14,19,25,26]. Except for the silver fox, these mutations are almost completely epistatic to the *agouti* gene, even in the heterozygous state.

In the present study we have identified two substitution mutations in the melanocortin 1 receptor (MC1R) in the blue fox, that co segregate completely with the blue fox phenotype in crosses between blue fox and winter white arctic fox. In addition to causing a uniform dark summer pelage, the two mutations also prevent the development of a completely white winter pelage. These findings indicate that the agouti/MC1R regulatory system is involved in seasonal changes in coat color found in arctic fox.

2. Materials and methods

2.1. Animals

The family study with farmed blue foxes was done based on a cross between a heterozygous female blue fox and a white male in the research farm at the Norwegian University of Life Sciences (Fig. 1). Among ten offspring, two were blue foxes and the remaining eight were of the winter white phenotype. In addition to the family study, blood samples were drawn from seven wild arctic foxes (of the white morph) that were caught near Ny-Ålesund (78°55′N, 11°56′E), Svalbard, Norway. These animals were a part of a larger project at the Norwegian Polar Institute, Tromsø, Norway [6,7]. Verification of the mutations found in the blue fox was also done in a blue fox originating from Finland.

2.2. PCR amplification and sequencing

DNA was isolated from blood samples following standard protocols. The complete coding region of the *MC1R* gene was amplified with primers AL1 and AL2 (Table 1). All primers are designed based on the red fox *MC1R* sequence (X90844). The PCR reaction was carried out in a 20 μl reaction containing 20 ng genomic DNA, 10 pmol of primers AL1 and AL2, 200 μM dNTP, standard buffer conditions and 1.5 U Taq polymerase. DNA was denatured for 3 min at 94 °C, and PCR run for 35 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. Sequencing of the complete region was carried out with

Table 1
Primers used in the study to amplify, sequence and genotype the arctic fox *MC1R* gene

Primer	Position	Direction	Sequence of primer in 5′–3′ direction
AL1	÷54 to ÷35	Forward	GAA CTG AGC GAG ACA CCT GA
AL2	980–997	Reverse	ATC ACC ACC TCC CTT TGC CCA
AL3	192–211	Forward	CAA GAA CCG CAA CCT GCA CT
AL4	42–61	Reverse	TGG TTG GGG AGG TGG CAA TG

Positions are given relatively to the translational start ATG of the red fox *MC1R* gene (X90844).

primers AL1, AL2 and AL3 using the protocol of Applied Biosystems.

2.3. RFLP analysis

Following the detection of two mutations in the *MC1R* gene of arctic fox, a RFLP reaction was developed to distinguish between the two variants. Shortly, primer AL1 and AL4 were used to amplify a 116 bp region containing the first of two linked mutations of interest localized to amino acid positions 5 and 280. PCR was carried out in an 20 μl reaction containing 10 pmol of each primer and 20 ng DNA, and was run for 35 cycles at 95 °C for 15 s, 60° for 15 s, and 72° for 15 s. The PCR fragment was cut with *ApaI* at 25 °C over night. If the wild type allele is present, the reaction will produce two fragments of 71 and 45 bp. If the blue fox allele is present, ApaI will not cut the fragment. The mutation localized to amino acid position 280 was genotyped by DNA-sequencing (Applied Biosystems) in the present study.

3. Results and Discussion

By sequencing the complete coding region of the *MC1R* gene of the winter white arctic fox (AJ786717), as well as the blue color variant (AJ786718), two mutations causing amino acid substitutions were discovered. In position 5, the glycin residue is substituted by a cysteine in the blue fox, while a phenylalanine is replaced by a cysteine at position 280. These two mutations are localized to the initial extracellular region of the receptor and the external boundary of transmembrane domain 7, respectively. A family study based on a cross between a heterozygous female blue fox and a white male fox (Fig. 1) verified that the two mutations cosegregated with the dominant blue phenotype. The wild arctic foxes from Svalbard have a *MC1R* genotype identical to the winter-white domestic arctic foxes from Norway, whereas a blue fox of Finnish origin have a *MC1R* genotype identical to the Norwegian blue fox.

Previously reported *MC1R* mutations causing dominant eumelanizing effects are localized within or close to the 2nd and 3rd transmembrane (TM) domains of the 317 amino acid long receptor [3,13]. However, since cysteine has the capability of forming disulfide bridges with other cysteine residues, a conformational change of the receptor with an effect comparable to the TM2/TM3 – mutations might be foreseen. Such interactions could be formed either between the two new cysteine residues, or to cysteine residues localized to any of the extracellular regions of the receptor. Both alterations in the *MC1R* gene of the blue fox are found within relatively well conserved amino acid positions of the receptor (Fig. 2). The 5th position is conserved in all mammalians characterized so far, except for mouse which has a glutamine substituting the glycine. In position 280, all species are identical except for pig which has a valine substituting the phenylalanine. Based on the present data, it is not possible to conclude whether one or both of these mutations are involved in the observed phenotypic change.

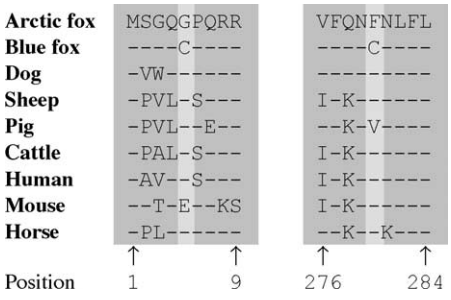

Fig. 2. Sequence comparison for *MC1R* regions that span amino acid 5 (amino acid 1–9 in the left box) and 280 (region 276–284, or 274–282 in mouse which has a two aa deletion). For the regions illustrated, additional species have been shown to be identical to species included in this figure [5,10–12,16,18,25,26].

The darker dorsal and lighter ventral pattern found in the summer pelage of the arctic fox is comparable to different *agouti* alleles found in mouse, affecting the dorsum and ventrum independently. This phenomenon is explained by alternative promoter use in the *agouti* gene [4,15,20,23]. In this respect, the blue fox mutation act as a classical constitutively activated *MC1R* mutation in being epistatic to the *agouti* gene. Inter-species crossing between the winter white arctic fox and the standard silver variant (*aaE*$^{+}$*E*$^{+}$) of the red fox (*Vulpes vulpes*) provide firm evidence for a functional agouti protein in the arctic fox. The standard silver fox do not carry a functional agouti protein, and the red color found in the offspring (named Golden island) must therefore be ascribed the functional "arctic" agouti protein [17,25].

Based on classical genetic studies [2] a hypothesis was put forward that a dominant *agouti* allele (A^{w}) is responsible for gradually eliminating all eumelanin from the birth coat of the pups and from the colored summer coat of adults, thus replacing the summer color by the white winter coat. A functional explanation of this could be that the equilibrium between the agouti protein and α-MSH is shifted according to the photoperiods, with a higher concentration of α-MSH during summer, and a relatively higher level of agouti protein during the winter season. According to this model one should expect a yellow/red winter coat instead of the observed white one. However, a general reduction in melanocyte activity

combined with a rapid growth of the winter coat may dilute the pheomelanin in a level giving this white appearance.

α-MSH and UV radiation are well known as major physiological signals that modulate skin pigmentation [1,22]. Melanocytes react to these signals by proliferation, increased dendriticity and elevated pigment synthesis. Prolonged day length during summer increases the UV radiation and the local α-MSH production in the skin is increased. Although the fox skin is not directly subjected to sunlight, it has been proposed that the hair shaft may function as a fiber-optic system for guiding UV light to the hair follicle [9]. This has of course to be verified experimentally, but should not yet be exclude as a possible contribution to the eumelanization seen in the summer pelage. In addition to a local reaction in the skin, due to increased day length, SCN guided signals do most likely play an important role in forming a coordinated reaction to seasonal changes. Changes in circulating levels of α-MSH synthesized in the pituitary gland is one possible SCN-guided signal.

Based on our results we do assume that the two mutations found in the *MC1R* gene of the blue fox are acting as a classical dominant mutation, causing a constitutive active receptor. Such mutations are capable of activating the receptor in the absence of the α-MSH ligand [14]. These processes may explain both the general darker pelage of the blue fox, the lack of a light ventrum and a lesser responsiveness to shifts in the agouti/α-MSH equilibrium influencing the seasonal changes in coat color.

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References

[1] Abdel-Naser MB, Krasagakis K, Garbe C, Eberle J. Direct effects on proliferation, antigen expression and melanin synthesis of cultured normal human melanocytes in response to UVB and UVA light. Photodermatol Photoimmunol Photomed 2003;19:122–7.
[2] Adalsteinsson S, Hersteinsson P, Gunnarsson E. Fox colors in relation to colors in mice and sheep. J Hered 1987;78:235–7.
[3] Andersson L. Melanocortin receptor variants with phenotypic effects in horse, pig and chicken. Ann NY Acad Sci 2003;994:313–8.
[4] Bultman SJ, Michaud EJ, Woychik RP. Molecular Characterization of the Mouse Agouti Locus. Cell 1992;71:1195–204.
[5] Everts RE, Rothuizen J, van Oost BA. Identification of a premature stop codon in the melanocyte-stimulating hormone receptor gene (MC1R) in Labrador and Golden retrievers with yellow coat color. Anim Genet 2000;31:194–9.
[6] Fuglei E, Øritsland NA. Seasonal trends in body mass, food intake and resting metabolic rate, and induction of metabolic depression in arctic foxes (*Alopex lagopus*) at Svalbard. J Comp Physiol [B] 1999;169:361–9.
[7] Fuglei E, Aanestad M, Berg JP. Hormones and metabolites of arctic foxes (*Alopex lagopus*) in response to seasonal variations, starvation and re-feeding. Comp Biochem Physiol A 2000;126:287–94.
[8] Hofman MA. The brain's calendar: neural mechanisms of seasonal timing. Biol Rev Camb Philos Soc 2004;79:61–77.
[9] Iyengar B. The hair follicle: a specialised UV receptor in the human skin? Biol Signals Recept 1998;7:188–94.
[10] Kijas JMH, Wales R, Törnsten A, Chardon P, Moller M, Andersson L. Melanocortin receptor 1 (MC1R) mutations and coat color in Pigs. Genetics 1998;150:1177–85.
[11] Klungland H, Våge DI, Gomez-Raya L, Adalsteinsson S, Lien S. The role of melanocyte-stimulating hormone (MSH) receptor in bovine coat color determination. Mamm Genome 1995;6:636–9.
[12] Klungland H, Røed K, Nesbø CL, Jakobsen K, Våge DI. Melanocyte stimulating hormone receptor (MC1R) gene as a tool for evolutionary studies in artiodactyles. Hereditas 1999;131:30–6.
[13] Klungland H, Våge DI. Molecular genetics of pigmentation in domestic animals. Curr Genome 2000;1:223–42.
[14] Lu D, Våge DI, Cone RD. A ligand-mimetic model for the constitutive activation of the melanocortin-1 receptor. Mol Endocrinol 1998;12:592–604.
[15] Millar SE, Miller MW, Stevens ME, Barsh GS. Expression and transgenic studies of the mouse agouti gene provide insight into the mechanisms by which mammalian coat color patterns are generated. Development 1995;121:3223–32.
[16] Mountjoy KG, Robbins LS, Mortrud MT, Cone RD. The cloning of a family of genes that encode the melanocortin receptors. Science 1992;257:1248–51.
[17] Nes N, Einarsson EJ, Lohi O. Beautiful fur animals and their color genetics. Oslo Norway: Scientifur; 1987.
[18] Rieder S, Taourit S, Mariat D, Langlois B, Guerin G. Mutations in the agouti (ASIP), the extension (MC1R), and the brown (TYRP1) loci and their association to coat color phenotypes in horses (Equus caballus). Mamm Genome 2001;12:450–5.
[19] Robbins LS, Nadeau JH, Johnson KR, Kelly MA, Roselli-Rehfuss L, Baack E, et al. Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. Cell 1993;72:827–34.
[20] Siracusa LD. The agouti gene: turned on to yellow. Trends Genet 1994;10:423–8.
[21] Underwood LS, Reynolds P. Photoperiod and fur lengths in the arctic fox (*Alopex lagopus*). Int J Biometeorol 1980;24:39–48.
[22] Virador VM, Muller J, Wu X, Abdel-Malek ZA, Yu ZX, Ferrans VJ, et al. Influence of alpha-melanocyte-stimulating hormone and ultraviolet radiation on the transfer of melanosomes to keratinocytes. FASEB J 2002 Jan;16:105–7.
[23] Vrieling H, Duhl DMJ, Millar SE, Miller KA, Barsh GS. Differences in dorsal and ventral pigmentation result from regional expression of the mouse agouti gene. Proc Natl Acad Sci USA 1994;91:5667–71.
[25] Våge DI, Lu D, Klungland H, Lien S, Adalsteinsson S, Cone RD. A non-epistatic interaction of agouti and extension in the fox Vulpes vulpes. Nat Genet 1997;15:311–5.
[26] Våge DI, Klungland H, Lu D, Cone RD. Molecular and pharmacological characterization of dominant black coat color in sheep. Mamm Genome 1999;10:39–43.