

Molecular and pharmacological characterization of dominant black coat color in sheep

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Abstract. Dominant black coat color in sheep is predicted to be caused by an allele E^D at the *extension* locus. Recent studies have shown that this gene encodes the melanocyte stimulating hormone receptor (MC1-R). In mouse and fox, naturally occurring mutations in the coding region of MC1-R produce a constitutively activated receptor that switches the synthesis from pheomelanin to eumelanin within the melanocyte, explaining the black coat color observed phenotypically. In the sheep, we have identified a Met→Lys mutation in position 73 (M73K) together with a Asp → Asn change at position 121 (D121N) showing complete cosegregation with dominant black coat color in a family lineage. Only the M73K mutation showed constitutive activation when introduced into the corresponding mouse receptor (mMC1-R) for pharmacological analysis; however, the position corresponding to D121 in the mouse receptor is required for high affinity ligand binding. The pharmacological profile of the M73K change is unique compared to the constitutively active E92K mutation in the sombre mouse and C123R mutation in the Alaska silver fox, indicating that the M73K change activates the receptor via a mechanism distinct from these previously characterized mutations.

Black coat color in sheep that is inherited in a dominant manner is known in a number of sheep breeds. Although it seems reasonable to assume that dominant black is caused by an allele at the *extension* locus (E^D), lack of the recessive *extension* allele (e) makes it complicated to establish the E series in sheep by classical means (Searle 1968). However, evidence for the presence of E^D and E^+ in French sheep breeds has been reported (Lauvergne 1975). More recently, molecular studies have shown that the *extension* locus encodes the melanocyte stimulating hormone receptor (MC1-R) (Mountjoy et al. 1992; Robbins et al. 1993). The MC1-R is a G protein-coupled membrane receptor that, when activated, modulates the switch from pheomelanin to eumelanin synthesis in the melanocyte.

Normally, MC1-R is activated by the binding of melanocyte stimulating hormone (α -MSH). However, it has recently been demonstrated in mouse and fox that naturally occurring mutations in the coding region of the MC1-R could give a constitutively activated receptor in the absence of any α -MSH stimulation when tested in cell lines expressing the mouse MC1 receptor (Robbins et al. 1993; Våge et al. 1997). This functional change in the receptor causes a similar intracellular response as α -MSH binding, and explains the dark coat color observed in animals carrying dominant mutations.

A second gene termed *agouti* is closely interacting with MC1-R. This gene encodes a 131 amino-acid protein that acts as an antagonist of MC1-R by blocking α -MSH stimulation (Bultman et al. 1992; Lu et al. 1994; Miller et al. 1993). Since constitutively

activating mutations in MC1-R are acting downstream of α -MSH stimulation by altering the receptor configuration itself, these are usually not antagonized by *agouti*. This is the functional explanation why dominant *extension* alleles tend to be epistatic even over top dominant *agouti*-alleles. In sheep, Lauvergne (1975) reported that dominant black was epistatic over all *agouti* alleles. This finding supports the hypothesis that dominant black in sheep is caused by a dominant *extension* allele (E^D).

In the present study, we describe the molecular and pharmacological nature of two naturally occurring mutations in the coding region of the sheep MC1-R that cosegregate with dominant black coat color. One amino acid change appears to activate the MC1-R via a mechanism not seen previously for this receptor (M73K), while a cosegregating change (D121N) may identify a residue critical for ligand binding. PCR-RFLP tests have been developed for both mutations.

Materials and methods

Animals. The animals used in this study are of the Norwegian Dala breed of sheep. These animals are normally white, but there are examples of entirely black individuals. As the animals get older, the wool of the black animals change into a more brownish color (Fig. 1A). The family material represent the first or second generation after a black ewe. Altogether, 22 animals were tested, 9 black and 13 white (Fig. 1B).

Cloning and sequencing. The complete MC1-R coding region of the one white and one black sheep was amplified by E1 and E2 (Fig. 2). These primers were designed based on the corresponding bovine sequence (Vanetti et al. 1994). Each PCR reaction was carried out in a total volume of 50 μ l, containing 200 ng genomic DNA, 25 pmol of each primer, 200 μ M of each dNTP, standard buffer conditions and 2.0 U Taq polymerase. Genomic DNA was denatured for 3 min at 95°C, and PCR run for 40 cycles at 95°C for 15 sec, 65°C for 30 sec, and 73°C for 60 sec. Primers E2, E4, E5, and E7 were used for direct sequencing of amplified DNA by using ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA). Sequencing of the 5' end was carried out using Dye Primer chemistry (Perkin Elmer).

PCR-RFLP at M73K. PCR amplifications were carried out with primers E3 and E6 in a volume of 20 μ l containing 50 ng DNA, 10 pmol of each primer, 200 μ M dNTP, standard buffer conditions and 1 U Taq polymerase. Genomic DNA was denatured for 3 min at 95°C, and PCR run for 35 cycles at 95°C for 15 sec, 60°C for 30 sec, and 73°C for 30 sec. Fifteen μ l of the PCR-product was digested with *NlaIII* (CATG/) for 2 hours at 37°C in a 20 μ l reaction. Digested DNA was separated on a 4% agarose gel. When the wild-type allele is present, the initial 300 bp fragment is cut by *NlaIII* into fragments of 118 bp, 90 bp, 48 bp, 27 bp, and 17 bp, respectively. If the M73K mutation is present, the PCR product is cut into fragments of 145 bp, 90 bp, 48 bp and 17 bp, respectively (Fig. 3A).

PCR RFLP at D121N. PCR amplifications were carried out with primers E3 and E8 in a volume of 20 μ l as described for the M73K polymor-

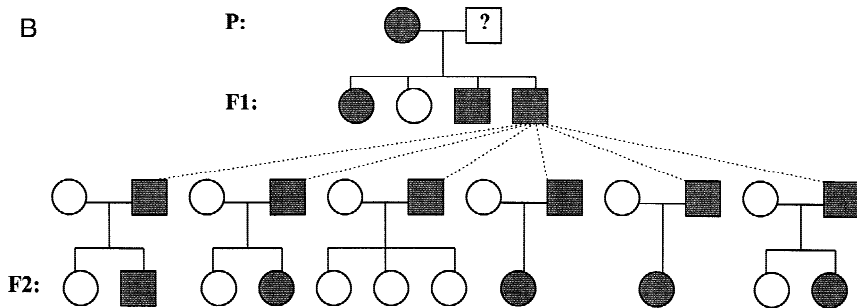


Fig. 1. (A) Animals used in this study are of the Norwegian Dala breed of sheep. These animals are normally white, but there are examples of entirely black animals. As the animals get older, the wool of the black animals change into a more brownish color. (B) The family material represents the first and second generation after a black ewe. Altogether, 22 animals were tested, 9 black and 13 white. Gray boxes indicate black animals, white boxes indicate white animals. Both M73K and D121N showed complete linkage with black coat color.

E1	M13-cat gcc tgg gcc gac att tgt	÷117- ÷97	forward
E2	ctc acc ttc agg gat ggt cta	1024-1043	reverse
E3	gtg cct gga ggt gtc cat c	102-120	forward
E4	caa gaa ccg caa cct gca ct	192-211	forward
E5	cca tgg tgt cca gcc tct	380-397	forward
E6	aag cag agg ctg gac acc at	382-401	reverse
E7	ctc ttc atc acc tac tac aa	532-551	forward
E8	ggc cag gaa gag gtt gaa g	837-855	reverse
E9	ata ctt ggg cga gtg cag gtt gc	208-230 (mouse)	reverse
E10	tac ttc atc tgc tgc ctg gcc	231-251 (mouse)	forward

phism, except that the annealing temperature was set to 59°C and the extension time to 45 sec. Fifteen μ l of the PCR-product was digested with *Mse*I (T/TAA) for 2 hours at 37°C in a 20 μ l reaction. Digested DNA was separated on a 2% agarose gel. When only the wild-type allele is present, the initial 754 bp fragment is not cut by *Mse*I. If the D121N mutation is present, the 754 bp fragment is cut into fragments of 258 bp and 496 bp, respectively (Fig. 3B).

Site-directed mutagenesis. The M73K and D121N mutations were introduced into the mouse MC1 receptor coding sequence by polymerase chain reaction. Since the sheep MC1-R is two amino acids longer than the mouse MC1-R, these two mutations are referred to as M71K and D119N, respectively, in the context of the mouse receptor. Two oligonucleotides were designed end to end to hybridize to opposite strands and amplify an entire pBS plasmid (Stratagene, La Jolla, CA) containing the complete mouse MC1-R coding sequence. One primer (E9) contained the M73K change and the other was complementary to the wild type receptor (E10). Following PCR using Vent polymerase (New England Biolabs, Beverly, MA), linear DNA were purified by agarose gel electrophoresis, ligated and clonally isolated. The mutation, as well as the remainder of the mMC1-R, were confirmed by sequencing using an ABI model sequencer. A previously made mutation pD119N was used as a template to make the M71K & D119N double mutation using primers E9 and E10 (Lu et al. 1998).

M73K

	69	70	71	72	73	74	75	76	77	78	79
Allele E ^r	:CTG	CAC	TCC	CCC	ATG	TAC	TTC	ATC	TGC	TGC	
Allele E ^p	:---	---	---	---	-A-	---	---	---	---	---	---
Allele E ^r	:Leu	His	Ser	Pro	Met	Tyr	Tyr	Phe	Ile	Cys	Cys
Allele E ^p	:---	---	---	---	Lys	---	---	---	---	---	---

D121N

	117	118	119	120	121	122	123	124	125	126	127
Allele E ^r	:GAC	AAT	GTC	ATT	GAC	GTG	CTC	ATC	TGC	AGC	TCC
Allele E ^p	:---	---	---	---	A-	---	---	---	---	---	---
Allele E ^r	:Asp	Asn	Val	Ile	Asp	Val	Leu	Ile	Cys	Ser	Ser
Allele E ^p	:---	---	---	---	Asn	---	---	---	---	---	---

Fig. 2. Mutations in the sheep MC1-R gene, showing the Met→Lys change at position 73 (M73K), and the Asp → Asn change at position 121 (D121N). The corresponding positions in the mouse sequence is 71 and 119, respectively. The complete coding sequence of the sheep MC1-R will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Y13965.

Receptor expression. The mutated MC1-R was subcloned into the pcDNA III vector (Invitrogen, San Diego, CA) for expression studies. Human embryonic kidney 293 cells were transfected with 20 μ g DNA, using the calcium phosphate method (Chen and Okayama 1987). Selection began 72 hours post-transfection in Dulbecco's modified Eagle's medium containing 10% newborn calf serum and 1 mg/ml geneticin.

β -galactosidase activity assay. HEK 293 stable cell lines expressing the wild type mMC1 receptor and the receptor mutants were transfected with a pCRE/ β -galactosidase (pCRE/ β -gal) construct using the calcium

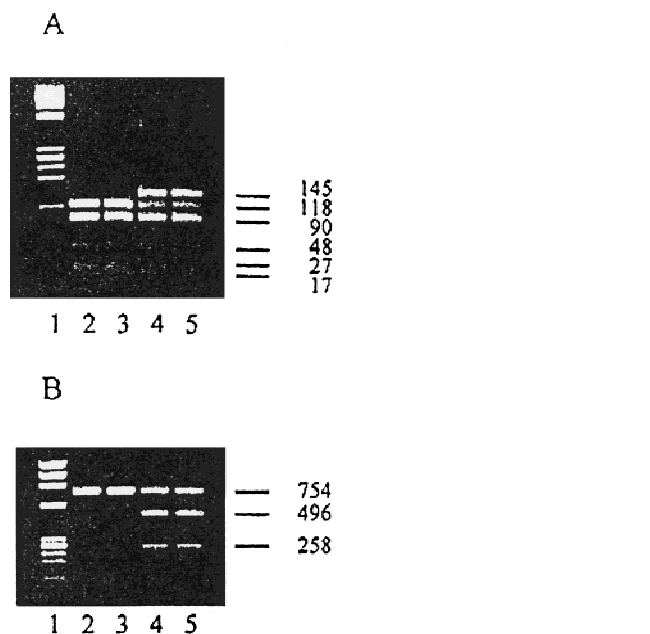


Fig. 3. (A) PCR-RFLP at M73K. Lane 1: ϕ X174 digested with *HaeIII*. Lanes 2 and 3: White animals (E^+E^+). Lanes 4 and 5: Black animals (E^+E^D). Molecular sizes are shown to the right. DNA is amplified with primers E3 and E6 and digested with *NlaIII*. When the wild-type allele is present, the initial 300 bp fragment is cut into fragments of 118 bp, 90 bp, 48 bp, 27 bp, and 17 bp, respectively. If the M73K mutation is present, the PCR product is cut into fragments of 145 bp, 90 bp, 48 bp, and 17 bp, respectively. (B) PCR RFLP at D121N. Lane 1: ϕ X174 digested with *HaeIII*. Lanes 2 and 3: White animals (E^+E^+). Lanes 4 and 5: Black animals (E^+E^D). Molecular sizes are shown to the right. DNA is amplified with primers E3 and E8 and digested with *MseI*. When only the wild-type allele is present, the initial 754 bp fragment is not cut by *MseI*. If the D121N mutation is present, the 754 bp fragment is cut into fragments of 258 bp and 496 bp, respectively.

phosphate method (Chen and Okayama 1987). 4 μ g of pCRE/ β -gal DNA was used for transfection of a 10-cm dish of cells. After 15 to 24 hours, cells were split into 96-well plates with 20,000 to 30,000 cells per well and incubated until 48 hours post transfection. Cells were then stimulated with different concentrations of α -MSH and Ac-[Nle⁴-D-Phe⁷]- α -MSH (NDP- α -MSH) diluted in stimulation medium (Dulbecco's modified Eagle's medium containing 0.1 mg/ml bovine serum albumin and 0.1 mM isobutylmethylxanthine) for 6 hours at 37°C in a 5% CO₂ incubator. Aliquots of cells were also stimulated by forskolin (10 μ M) to normalize for transfection efficiency. After stimulation, cells were lysed in 50 μ l lysis buffer (250 mM Tris-HCl, pH 8.0, 0.1% Triton X-100), frozen and thawed, and then assayed for β -galactosidase activity as described (Chen et al. 1995). β -galactosidase activity was normalized both to protein concentrations and to forskolin (10 μ M) stimulated level. Data represent means and standard error from triplicate data points and curves were fitted by nonlinear regression using Prism software (Graphpad).

Ligand binding. Competition binding experiments were performed on stable cell lines containing the wild type mMC1 receptor or mutant receptors. Those cells were plated at 5×10^6 cells per well to a 24-well plate the day before the binding experiment was performed. The cells were then incubated for 45 minutes at room temperature in binding medium (1 mg/ml BSA in Ca⁺⁺/Mg⁺⁺ PBS) containing 30–100,000 cpm of ¹²⁵I-NDP- α -MSH per well. Series concentrations of unlabeled α -MSH (Fig. 4C) or NDP- α -MSH and α -MSH (Fig. 4D) were used as indicated to compete with the labeled NDP- α -MSH. Controls of non-specific binding contain 1 or 10 μ M of unlabeled α -MSH. After 45 minutes of incubation, the medium was aspirated and the cells were washed once with 1 ml of BSA/PBS (1 mg/ml BSA in Ca⁺⁺/Mg⁺⁺ PBS) per well. Later, 0.5 ml of Gibco's versin was used to transfer cells to test tubes for counting radioactivity. Data represent means and standard deviation from duplicate data points and curves were fitted by nonlinear regression using Prism software (Graphpad).

Results

We have amplified and sequenced the complete coding region of the MC1-R gene in two sheep of the Norwegian Dala breed. One of the animals was completely white, while the other was black (Fig. 1A). In both animals we found one continuous open reading frame (ORF) containing two additional amino acids compared to the corresponding mouse sequence (Mountjoy et al. 1992). In the black animal two replacement mutations were identified, a Met \rightarrow Lys at position 73 (M73K) and an Asp \rightarrow Asn at position 121 (D121N) (Fig. 2). Both mutations could easily be tested by PCR-RFLP using the restriction enzymes *NlaIII* (M73K) and *MseI* (D121N) (Fig. 3A–B). The inheritance of these two mutations were tested in a family comprising 22 animals segregating black coat color (Fig. 1B). Both mutations showed complete linkage with black coat color. The fact that the black animals were heterozygous at these two positions (73, 121), while the white animals were homozygous, is supporting our assumption of dominant inheritance of the black coat color.

Both M73K and D121N were introduced into mMC1-R to test the properties of the mutant receptors, since extensive pharmacological data exist already for the mouse receptor. The pharmacological analysis revealed that the D119N mutation alone in the context of the mouse MC1-R did not constitutively activate the receptor. However, replacement of the aspartate residue at this position with lysine, asparagine, or valine decreases ligand binding affinity 10–100 fold (Lu et al. 1998). Consequently, we tested the effect of M71K alone, and M71K plus D119N together in the mMC1-R. The M71K change alone potently activated the MC1-R to approximately 40% of maximal stimulation levels in the absence of ligand. Additionally, this receptor could be further activated to maximal levels by either α -MSH or NDP- α -MSH (Fig. 4A–B). Competition binding studies showed that the IC₅₀ for the M71K receptor was approximately four times lower than that of the wild type receptor, indicative of an increased affinity for ligand (Fig. 4C). Introduction of the D119N change back into the M71K single mutant resulted in a receptor with properties more reminiscent of the mutations found in mouse and fox, in that mutations in these receptors reduced ligand efficacy. The addition of the D119N change reduced the extent of constitutive activation of the receptor, reduced the efficacy of NDP- α -MSH 3-fold, and reduced the efficacy of α -MSH to undetectable levels (Fig. 4A,B). Furthermore, while ligand was capable of activating the double mutant at sufficiently high concentrations, binding to this receptor could not be detected using the less sensitive assay of ligand binding (Fig. 4D).

Discussion

Molecular studies in mouse, cattle, horse, and fox have shown that most of the naturally occurring *extension* alleles are caused by mutations within the coding region of MC1-R, and the dominant acting mutations have specifically been localized within, or in close proximity to the extracellular portion of the 2nd and 3rd transmembrane region (TM2 and TM3) (Robbins et al. 1993; Klungland et al. 1995; Marklund et al. 1996; Våge et al. 1997). In the sheep, M73K is localized close to TM2 whereas D121N is found within TM3. Additionally, both mutations cosegregated completely with black coat color in the family material. Based on the segregation study and the localization, both M73K and D121N (or the combination of the two) could explain the dominant black coat color in sheep.

In order to further analyze these results, both mutations were introduced into the corresponding positions in the mouse MC1 receptor for pharmacological analysis. The MC1-R is highly conserved, showing 84% amino acid similarity when comparing the sheep and mouse sequence (Mountjoy et al. 1992). It was then revealed that the M71K mutation (M73K in sheep) alone was able to constitutively activate the receptor, while the D119N mutation

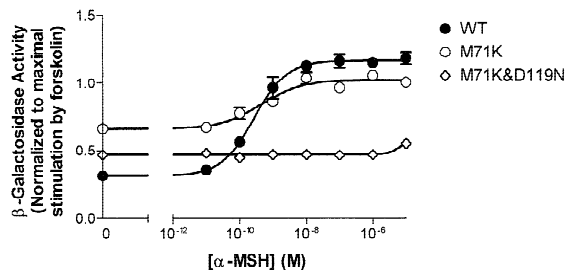


Figure 4A

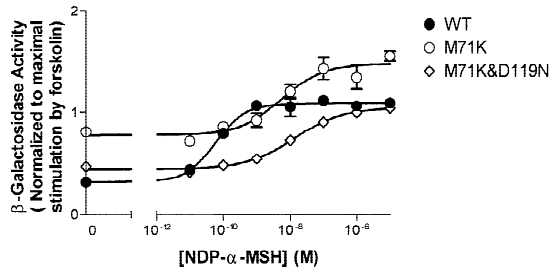


Figure 4B

Fig. 4. Pharmacology of the M71K and M71K with D119N of the mouse MC1-R. (A) α -MSH stimulation curves for the mouse MC1-R wild type and M71K mutants. Both mutants result in constitutive activation of the receptor. Wild type and mutant MC1 receptors are assayed by analyzing their ability to activate expression of a cAMP-induced β -galactosidase fusion gene. Cells stably expressing each receptor and transiently expressing the fusion construct were stimulated for 6 h with medium alone, $10 \mu\text{M}$ forskolin, or increasing concentrations of α -MSH (A), or NDP- α -MSH (B), then β -galactosidase concentrations were determined. Data points represent means of triplicate determinations divided by maximal levels of β -galactosidase activity achieved by $10 \mu\text{M}$ forskolin stimulation to normalize for transfection efficiency, and error bars indicate standard deviations. (B) NDP- α -MSH stimulation curves for the mouse MC1-R wild type

(D121N in sheep) did not have this ability. Unlike most of the naturally occurring MC1-receptor mutations, including E92K and L98P mutations found in the mouse (Robbins et al. 1993; Cone et al. 1996), as well as the C123R mutation found in the fox (Våge et al. 1997), M71K constitutively activates the receptor with slightly increased ligand affinity, while most other MC1-R mutants constitutively activate the receptors with decreased ligand efficacy and affinity. One mMC1 receptor mutation found in the tobacco mice, S69L may have a similar pharmacological property as M71K in receptor activation (Robbins et al. 1993); however, this receptor has not yet been characterized in detail. Considering the different location and different pharmacological profiles of the M71 and S69 mutations with E92K, L98P and C123R mutations, different mechanisms for constitutive activation are indicated.

In recent studies, Lu et al. proposed a ligand-mimetic model for constitutive activation of the MC1 receptor, which explains the constitutive activation of the receptor with lower ligand efficacy and affinity (Lu et al. 1998). These mutations transform the receptor into its active form, R^* , not by disrupting the internal constraint as proposed by rhodopsin studies (Robinson et al. 1992; Cohen et al. 1993) and the ternary allosteric model (Lefkowitz et al. 1993), but by indirectly mimicking ligand binding (Lu et al. 1998). Given the fact that both M71 and S69 are located close to the first intracellular loop of the receptor, a region indicated to affect the protonation state of the aspartate residue in the DRY sequence involved in G-protein coupled receptor activation, the mechanism for constitutive activation of M71K is probably similar to the other constitutive active mutations studied in the adrenergic

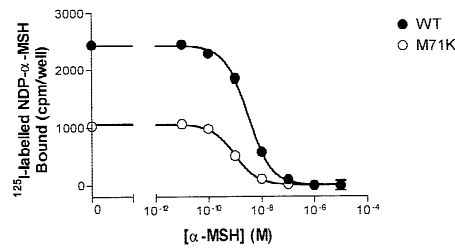


Figure 4C

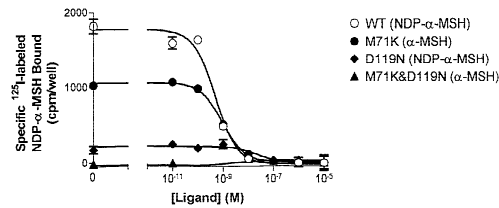


Figure 4D

and M71K mutants. Superpotent ligand NDP- α -MSH further activates the M71K with D119N mutant to levels equivalent to maximal stimulation of the wild type receptor by 10^{-6} M NDP- α -MSH. Methods are as in A, above. (C, D) Competition binding curves for the mouse MC1-R M71K and M71K with D119N mutants. Series concentrations of unlabeled α -MSH (C) or NDP- α -MSH and α -MSH (D) were used as indicated to compete with the labeled NDP- α -MSH. Conclusions about the relative IC_{50} values were only made when the same cold competitor was used (C). Nonspecific binding is determined as the counts bound in the presence of 10^{-5} M cold α -MSH. Data are displayed as specific binding. Data points represent means of duplicate determinations and bars indicate standard deviations.

receptors. That is, the M71K mutant probably activates the receptor by increasing the stability of the high affinity activated state of the receptor. Since studies of the mMC1-R indicated that D119 position (D121 in sheep) is involved in the high affinity ligand binding (Lu et al. 1998), it was not surprising that the M71K and D119N double mutant showed no detectable ligand binding.

When M71K and D119N were introduced together (double mutant), the extent of constitutive activation of the receptor was lowered and the efficacy of α -MSH was reduced to undetectable levels. This negative effect on receptor activation could indicate that the D119N change is not critical for the black coat color observed in sheep, and that M71K alone could give a similar phenotype. The loss of ligand affinity is no longer a factor in receptor function, however, given that the receptor is already constitutively active. In the context of the melanocyte, the decrease in ligand binding affinity could impact the regulation of the receptor (e.g., desensitization).

In the sheep, several *agouti* alleles have been reported including white, which is suggested to be the top dominant allele (Adalsteinsson 1970). *Agouti* alleles would normally be phenotypically expressed only in the presence of the wild type allele (E^+) at the *extension* locus. The observed ORF in the white animal in this study might therefore represent a functional E^+ allele, although this remains to be proved.

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