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Mutation in the Melanocortin 1 Receptor (MC1R) is associated with amber colour in the Norwegian Forest Cat

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Keywords:	Norwegian Forest Cat, MC1-R, amber, mutation, 3D model

Mutation in the Melanocortin 1 Receptor (MC1-R) is associated with amber colour in the Norwegian Forest Cat

Running title : MC1R mutation in domestic cats

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Abstract

Amber (previously called X-Colour) is a yellow recessive coat colour observed in the Norwegian Forest Cat population and apparently absent in other cat breeds. Until now there has never been any scientific evidence of yellow recessive mutation (*e*) reported in the *extension* gene in Felidae.

We sequenced the complete coding sequence region of the *melanocortin 1 receptor* in 12 amber, 3 carriers, 2 wild-type Norwegian Forest Cats (NFC), 1 wild-type European Shorthair and 2 "golden" Siberian cats and identified two single nucleotide polymorphisms: a non-synonymous mutation (FM180571: c.250G>A) and a synonymous (FM180571: c.840T>C). The c.250G>A SNP, further genotyped on 56 cats using PCR-RFLP, is associated with amber colour and only present in the amber cat lineages. It replaced an aspartic acid with a neutral polar asparagine in the second transmembrane helix (p.Asp84Asn), a position where *e* mutations have already been described. 3D models were built and showed electrostatic potential modification in the mutant receptor.

With these results and together with scientific literature, we can conclude that amber colour in Norwegian Forest Cats is caused by a single *MC1R* allele called *e* which has never been documented.

Keywords: Norwegian Forest Cat, MC1-R, amber, mutation, 3D model

Main text

The amber colour, initially called X-Colour, was officially reported in 1992 in the Norwegian Forest Cat (NFC) population and was never documented in other feline breeds. All amber cats have descended from a single ancestor, Kløfterhagens Babuschka, born in Norway in 1981, and this dame transmitted the amber trait to three daughters (Figure 1A). Amber NFC genealogies, partially represented in figure 1, show that non-amber cats can father amber kittens and amber matings only give amber kittens. There isn't any correlation between amber inheritance and the sex, supporting this colour as an autosomal recessive trait (Table S1).

Amber cats testing for the *brown* gene showed that they are genetically black (*B/B*) and confirmed the first test-mating results, which excluded the *chocolate* (*b*) *and cinnamon* (b^l) alleles and a new mutation in the *brown* gene, but also excluded the *burmese* (c^b), *siamese* (c^s), *albinos* (*c*) alleles and a new mutation in the *colour* gene (Utescheny & Langewische 2004). The amber colouration has been introduced onto different NFC coat colour and coat pattern backgrounds enabling a large colour variability: amber tabby (Figures 2A & 2B) with the three patterns, ticked (T^a), mackerel (T^m) and blotched (t^b), or amber non-agouti (solid) with ghost tabby pattern (Figures 2C & 2D). These patterns progressively brighten and almost totally disappear in amber solid AND tabby adults. Amber solid cats have dark paw pads and dark leather nose (Figure 2D), in contrast to pink-nosed amber tabby cats (Figure 2E) with pink paw pads at birth which darken afterwards; there are not any white marks in these body region. These observations were confirmed by testing amber NFC for the *agouti* allelic series. Amber colour also exists in dilute (*d*) (Figures 2A & 2E), silver (*A/-, I/-*) (Figure 2E) or smoke (*a/a, I/-*), eventually in tortoiseshell (*O/o*) (Figures 2F & 2G), possibly with white (*S*) (Figure 2B). Age-dependent colour maturation is clearly surprising: all kittens look like brown tabby or blue tabby for the dilute coat (Figures 2A & 2C), and then their original colour brightens and adults show an apricot/cinnamon-like colour (Figures 2B & 2D) or pinkish beige/fawn-like colour, called amber light (Figure 2E) with a few dark hairs on the back and tail (Figure 2B) and dark eye rims. Amber tortoiseshell female kittens present distinct black and red regions (Figure 2F), then black hairs become apricot and red hairs remain unchanged in adults (Figure 2G). A mating between an amber tortoiseshell dame and an amber sire gave two amber females and two red males (see cats R1 and R2 in figure 1C). This result proves that the *orange* allele is epistatic to amber, because these six cats are all homozygous for the amber allele, including the two red male kittens. Therefore, the amber pigment is

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different from the trichochrome red pigment, and is probably another sulphur-enriched pigment (yellow phaeomelanin) which seems to replace most of the hair eumelanin black pigment.

Diversity in mammalian pigmentation is achieved by differential expression and regional distribution of two pigment types: black eumelanin and yellow phaeomelanin. Switching between both syntheses is regulated by a paracrine signalling molecule, the agouti protein acting as an antagonist for the melanocortin 1 receptor (MC1-R). MC1-R is a seven transmembrane protein encoded by the *extension* gene, expressed on melanocytes and enabling eumelanin synthesis due to alpha-melanocyte stimulating hormone (α-MSH) (Robbins *et al.* 1993). In mammals, *extension* mutations causing constitutively active receptors (E^D) are dominant over the wild-type allele (E^+) and produce black coat, in contrast to inactivating recessive mutations (*e*), which result in yellow pigmentation (Klungland & Våge 2003). These inactivating *e* mutations enable a large colour variability from the "Kermode" black bear white-phased coat (Ritland *et al.* 2001) to the mouse tawny coat (Jackson 1994) and red coat possibly observed in dogs, humans (Rees 2003), pigs, chickens and horses (Andersson 2003). Such *e* mutation has never been described in Felidae whereas dominant E^D mutations are known in jaguar and jaguarundi (Eizirik *et al.* 2003) and are supposed to have existed in domestic cat (Vella *et al*. 1999).

As it is a yellow recessive coat colour, we hypothesized that this new colour in NFC could be the first mutation in the feline *extension* gene, coding for the MC1-R. Moreover, the yellow recessive mutation is only expressed in follicular melanocytes and has no consequence on epidermal melanocytes in dogs (Schmutz *et al.* 2002), as observed in amber cats (e.g. dark paw pads).

We worked with 3 wild-type cats (2 NFC and 1 European Shorthair), 33 amber NFC, 36 carrier NFC and 4 "golden" Siberian cats. Genomic DNA was extracted either with NucleoSpin Blood Quick Pure® kit (blood samples) or NucleoSpin XS Tissue® kit (hair samples) (Macherey Nagel). We sequenced the *MC1R* complete coding sequence region (954bp) in 12 amber, 3 carriers, 2 wild-type Norwegian Forest Cats, 1 wild-type European Shorthair and 2 "golden" Siberian cats after PCR amplification. The *MC1R* gene sequencing displayed in all sequences the same silent single nucleotide polymorphism (SNP) FM180571: c.840T>C in relation to Felis catus wild-type *MC1R* gene (AY237395). We also identified a non-synonymous FM180571: c.250G>A, only detected in cats from amber lineages. SNP c.250G>A was then genotyped on 56 additional cat samples (54 NFC and 2 "golden" Siberian cats) by RFLP-PCR using BstXI (Fermentas) and Hpy188I (New England Biolabs) which cleave the c.250A and the c.250G alleles.

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respectively. Primers forward (5'-TGCTGGGCTCCCTCAACTC-3') and reverse (5'-CCAGCACGTCAATGATGTCG-3') were designed to amplify a 342bp fragment (29 to 370). Amber cats were all homozygous c.250AA whereas carriers were all heterozygous c.250GA. This mutation associated with the amber colour in NFC has been called *e*.

Eizirik *et al*. (2003) sequenced the *MC1R* gene from 43 various breed cats. All had the same gene sequence (AY237395), including cats coming from European breeds and mainly NFC. Nevertheless, the silent c.840T>C SNP could be widespread in European cats and this warrants further phylogenetic analysis. The c.250G>A mutation replaces an aspartic acid at position 84 with an asparagine (p.Asp84Asn) and showed complete linkage with amber colour AND amber carrier cats, from all amber European lineages (Figure 1), some of which were related to the first amber NFC. Similar missense substitutions have already been described in humans, (p.Asp84Glu) associated with red hair (Valverde *et al.* 1995), and in horses, (p.Ser83Phe) coding for the chestnut coat (Marklund *et al.* 1996). Both mutations destabilize the alpha-helix structure in the fundamental second transmembrane field whose amino acid sequence is well-conserved among the MC1-R from different species (Figure 3). The human p.Asp84Glu variant was reported in several *MC1R* sequencing studies but with discrepant findings, because it was not always significantly associated with red hair in some studies (Rees 2003). Nevertheless, the mutant p.Asp84Glu shows *in vitro* a slightly impaired ability to bind the α-MSH (10-fold lower) and a much lower response to the melanocortin, as the maximum response is only 15% of the wild-type MC1-R, proving that this variant acts as a loss-of-function mutation (Ringholm *et al.* 2004). Even though the p.Asp84Glu mutant is known for a predisposition to skin cancers in humans, this effect probably does not exist in the amber cats. Indeed, the feline p.Asp84Asn mutation effects are only observed in the cat's coat, contrary to the human p.Asp84Glu mutant which associates red hair and fair skin (Rees 2003).

The aspartate present at position 83 in the Bovine Rhodopsin interacts with other conserved amino acids common to the Rhodopsin related G Protein-Coupled Receptors, forming a hydrogen binding network. This network extends in the binding pocket and has an important structural stabilizing role, and indeed a receptor activation role (Li *et al.* 2004). An alignment, performed on all MC1-R sequences from different species available in the protein database (more than 200, data not shown), indicates that this aspartic residue is also conserved in all sequences as well as in many melanocortin receptors (Figure 3). In order to check the impact of the p.Asp84Asn mutation, 3D models were built on the Geno3D server (Combet *et al.*

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2002) using 2rh1 as the template. The alignment (not shown) exhibited a 30% identity, making the modelling reliable. The comparison between the electrostatic potentials on the surfaces of the wild-type (Figure 4A) and the mutant (Figure 4B) models shows an important change at the bottom of the pocket. The wild-type pocket exhibits a greater negative potential (red patches) than the mutant. In cats, this change in the receptor binding moiety may explain the expected decrease in affinity for the binding of the positively charged α-MSH. In contrast, the human p.Asp84Glu mutation preserves the electrostatic properties but adds a carbon on the sidechain that may cause steric hindrance.

Models representing the interactions between α-MSH and human MC1-R have already been built and have emphasized the importance of the electrostatic potential for the binding. This field delimits an acidic pocket between the glutamate 94 and the aspartates 117 and 121 which interact with the arginine 8 from the α-MSH (Yang *et al.* 1997). These three acidic residues (Glu94, Asp117 and Asp121) are well-conserved among the melanocortin receptor amino acid sequences from all species (Figure 3). Furthermore, our 3D mutant model suggests that the acidic residue at position 84 also interacts in this binding, although the p.Asp84Asn replaces a negatively charged residue with a neutral polar one and thus only partially affects the α-MSH binding. This could explain why eumelanogenesis is incompletely inhibited in amber NFC, as compared to *e* mutations in adult mice (Robbins *et al.* 1993) and in other species where no black hair is observed. Another hypothesis would be that the aspartic acid 84 is functionally less critical for ligand binding than the third previous residues (Glu94, Asp117 and Asp121). This MC1-R region is of great interest for understanding the receptor behaviour, because each mutation can have opposite consequences according to its electrostatic modification. Indeed, the p.Glu92Lys (murine Glu92 is equivalent to Glu94 in the human and feline MC1-R, Figure 3) was initially reported in mice to be a constitutive active mutation which codes for a dominant black coat (Robbins *et al.* 1993), in contrast to the feline p.Asp84Asn substitution, which is associated with a yellow colour. Thus, the murine p.Glu92Lys introduces a positive charge instead of the negative aspartic acid and inhibits the α-MSH binding, but also causes constitutive activation by mimicking effects of the arginine ligand on the binding pocket conformation (Lu *et al.* 1998).

As observed in dogs (Rees 2003) and in horses (Andersson 2003), the feline *e* mutation enables a large range of yellow colour, from tawny (Figure 2G) to red-apricot (Figure 2D). In amber cats, this variability could be due to the rufism modifiers which have already been reported in the non-amber colours and which contribute to giving a wide range of expression of yellow pigmentation (Vella *et al.* 1999).

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Epistatic effects from inactivating recessive mutation *e* were firstly reported in mice (Robbins *et al.* 1993), but seem to be quite different than the *e* mutation in cats, since this epistasy is not observed in amber kittens. Adult amber coats are apricot and the tabby pattern is very faint whatever the genotype for the *agouti* gene, showing an epistasy from the *e* mutation to the *agouti* allelic series only in adult cats.

Incomplete epistasy of the fox E^A mutation to the *agouti* alleles was reported by Våge *et al.* (1997), but partial epistasy of an *e* mutation has never been shown in the animal kingdom as far as we are aware. This difference may be explained by the feline specific *tabby* gene which determines agouti hair only in the areas between tabby stripes. In amber kittens, agouti hairs are already apricot (Figure 2A) with a black tip, whereas non-agouti hairs are initially black and become apricot afterwards (Figure 2D).

It has also been hypothesized that body parts had different thresholds for the switch between the MC1-R and the agouti protein. The facial area has most likely got a low threshold for this switch (Schmutz *et al.* 2003) and this would explain why this region is the last region to brighten in amber solid cats, except for the nose in which epidermal melanocytes are not affected by the inactivating amber *e* mutation (Figure 2D).

Finally, we also studied *MC1R* gene in 4 "golden" Siberian cats: their colour is close to amber and the first Siberian and Norwegian cats originated from the same part of the world and so probably share common ancestors. The Siberian *MC1R* sequence has the same silent c.840T>C SNP but does not contain the c.250G>A. Thus, the "golden" colour from the Siberian cats is also genetically different from the amber colour. Further studies would be of great interest to elucidate if amber is really only specific to the NFC breed.

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Legends to figures

Figure 1: Pedigree analysis of amber NFC lineages

A - Swedish lineage; B – Dutch lineage; C – German lineage.
Circles represent females, squares represent males.
Isis, DeaDia and Froy Sparetta av Aesene are daughters from the first amber carrier, Kløfterhagens Babuschka. These 3 daughters were very probably amber carriers.
Numbers in symbols represent the same cats in each lineage.
Half-coloured symbols represent amber carriers cats, coloured symbols represent homozygous amber cats. Wild-type non-amber carrier cats have not been represented for simplification.
In figure C, R1 and R2 cats are phenotypically red. Their parents are amber homozygous c.250AA, the mother (N°16) is an amber tortoiseshell dame.
¥ signs represent cats whose *MC1R* was sequenced (15 animals).
* signs represent certain cats whose *MC1R* was genotyped by PCR-RFLP (13 animals), other tested cats are related to those ones.

Figure 2: Photographs and selected genotypes illustrating the variety of amber colours in the Norwegian Forest Cats

(A) A 4 week-olde litter of 3 amber blotched tabby (*), *A/-, D/-, i/i, o/o, s/s,* t^b/t^b and 4 light amber blotched tabby kittens (#), *A/-, d/d, i/i, o/o, s/s,* t^b/t^b. Areas between the black / blue tabby markings are brown to apricot-coloured (*) / grey to beige (#).
(B) Amber blotched tabby and white female at 16 months old, *A/-, D/-, i/i, o/o, S/-,* t^b/t^b. She has an apricot-coloured coat with a tabby pattern toning down and a few remaining black hairs on the tail (as pictures D & E). Her nose and paw pads are pink because of white marks.
(C) Amber solid 8 week-old kitten with dark nose, dark paw pads and ghost tabby pattern, *a/a, D/-, i/i, o/o, s/s, -/-*.
(D) Same cat as picture C at 9 months old: note the tabby pattern toning down to an apricot-coloured coat with dark paw pads and nose.
(E) Amber light silver mackerel tabby female with little white on the breast at 10 months old, *A/-, d/d, I/-, o/o, S/-,* $T^m/-$. She has a light pinkish-beige colour and her tabby pattern is already toned down.
(F) Amber silver tortoiseshell mackerel tabby and white female at 8 weeks old, *A/-, D/-, I/-, O/o, S/-,* $T^m/-$. Differentiation between orange (e.g. left forelimb) and amber is still easy.
(G) Same cat as picture F at 12 months old: dark stripes brightened and became tawny, mistakable for orange areas (e.g. right backlimb).

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Figure 3: Alignment of the protein region encompassing the MC1-R second transmembrane segment for 22 Rhodopsin related G Protein-Coupled Receptors
The multiple alignment was performed with the complete sequence by using CLUSTALW with default parameters
TM2 corresponds to the second transmembrane field amino acid sequence (residues 75 to 100).

TM3 corresponds to the third transmembrane field amino acid sequence (residues 114 to 144) (Ringholm *et al.*, 2004).
X, identity in most conserved residues in relation to the melanocortin 1 receptor sequences.
D represents Asp84; E represents Glu94; D represents Asp117 & Asp121 according to the MC1-R feline sequence (Q865E9).
Accession numbers in the protein database: MC1-R from cat (Q865E9), human (Q01726), mouse (Q01727), horse (P79166), wild boar (Q9TU05), rabbit (CAJ57384), cattle (NP_776533), sheep (CAA74298), dog (AAC33737), red fox (CAA62349), chicken (BAD91484), legless lizard (AAT90151) and zebrafish (NP_851301); MC2-R from human (Q01718) and mouse (NP_032586); MC3-R from human (NP_063941) and mouse (NP_032587); MC4-R from human (P32245) and mouse (P56450); MC5-R from human (P33032) and mouse (P41149); Bovine Rhodopsin (NP_001014890).

Figure 4: **3D models of wild-type and mutant melanocortin 1 receptors**

The 3D models of transmembrane moities of wild-type (A) and p.Asp84Asn mutant MC1-R (B) were built with geno3D server by using the beta-2-adrenergic receptor (2rh1 PDB code) as a template. The electrostatic potential was calculated with Delphi (Rocchia *et al.* 2001). The molecular surface was generated by using the MSMS program (Sanner *et al.* 1996). The orientation is top-down regarding the ligand binding site pocket.

Supplementary Material

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Table S1: Table of breeding types presenting the number of cats produced with and without the amber phenotype.

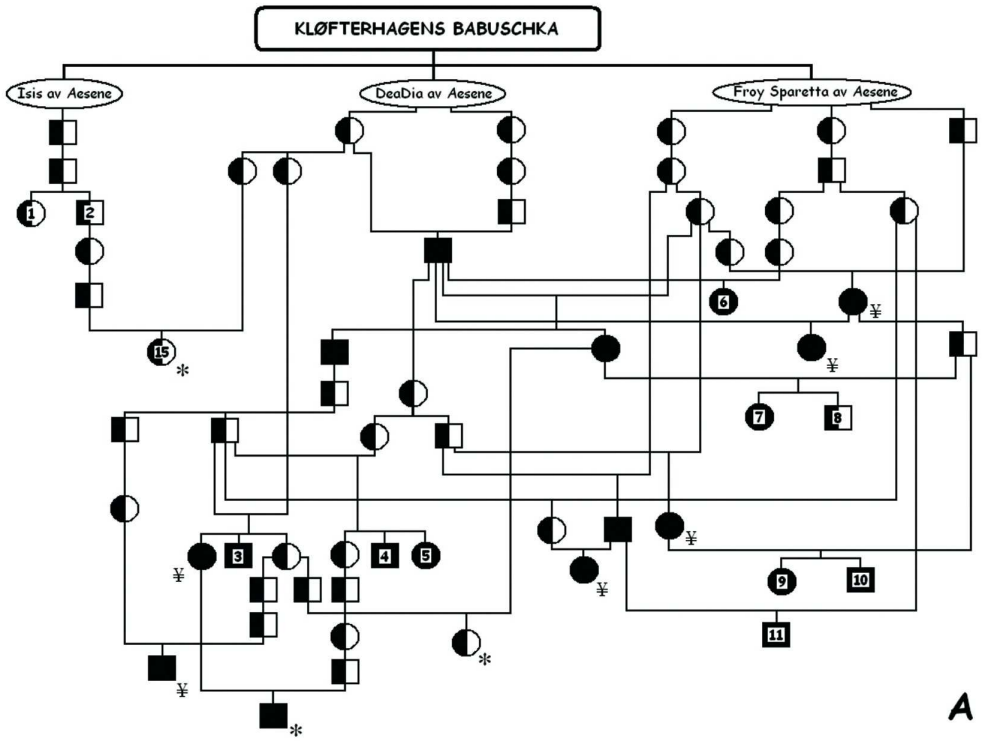

Figure 1A : Pedigree analysis of amber NFC Swedish lineage Circles represent females, squares represent males. Isis, DeaDia and Froy Sparetta av Aesene are daughters from the first amber carrier, Kløfterhagens Babuschka. These 3 daughters were very probably amber carriers. Numbers in symbols represent the same cats in each lineage. Half-coloured symbols represent amber carriers cats, coloured symbols represent homozygous amber cats. Wild-type non-amber carrier cats have not been represented for simplification. In figure C, R1 and R2 cats are phenotypically red. Their parents are amber homozygous c.250AA, the mother (N°16) is an amber tortoiseshell dame. ¥ signs represent cats whose MC1R was sequenced (15 animals). * signs represent certain cats whose MC1R was genotyped by PCR-RFLP (13 animals), other tested cats are related to those ones.
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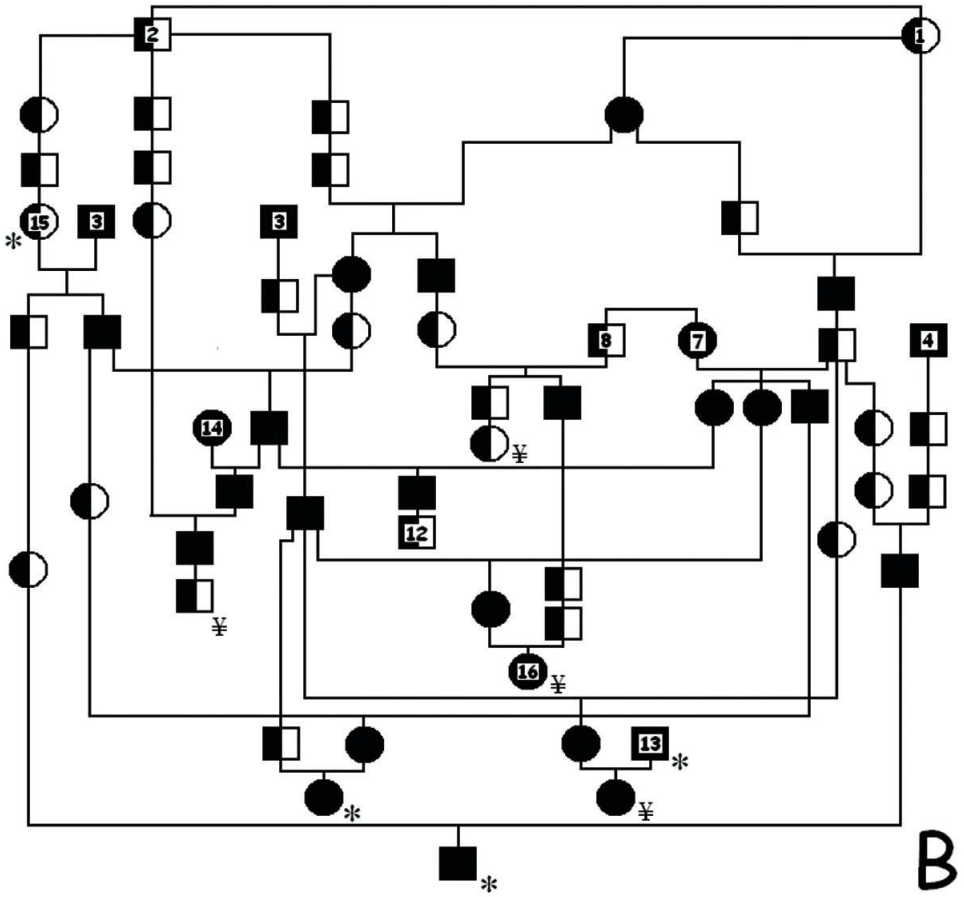

Figure 1B: Pedigree analysis of amber NFC Dutch lineage
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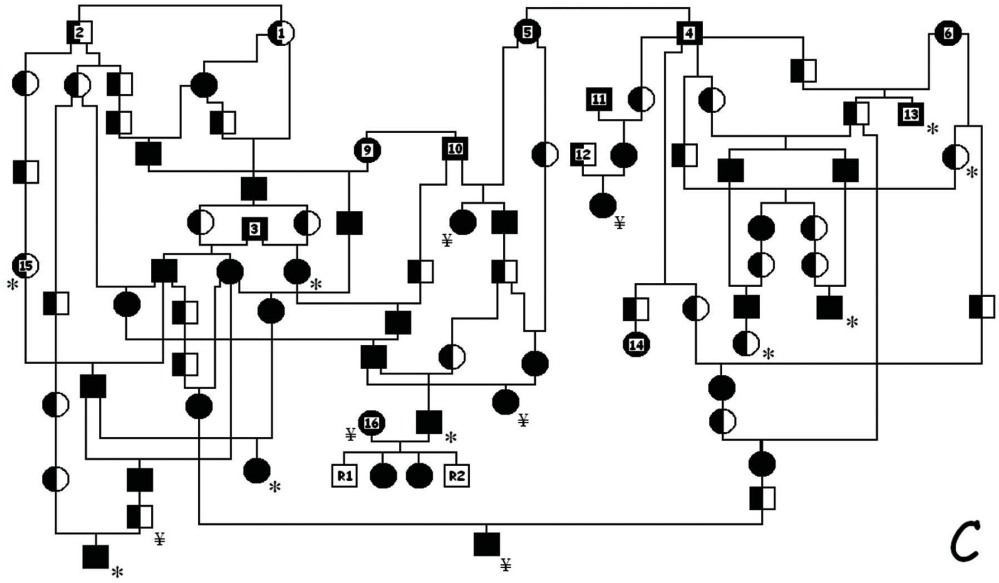

Figure 1C: Pedigree analysis of amber NFC German lineage
303x178mm (150 x 150 DPI)

Figure 2A: A 4 weeks-olded litter constituted of 3 amber blotched tabby (*), A/-, D/-, i/i, o/o, s/s, tb/tb and 4 light amber blotched tabby kittens (#), A/-, d/d, i/i, o/o, s/s, tb/tb. Areas between the black / blue tabby markings are brown to apricot-coloured (*) / grey to beige (#).
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Figure 2B : Amber blotched tabby and white female at 16 months old, A/-, D/-, i/i, o/o, S/-, tb/tb. She has an apricot-coloured coat with a tabby pattern toning down and a few remaining black hairs on the tail (as pictures D & E). Her nose and paw pads are pink because of white marks.
48x31mm (300 x 300 DPI)

Figure 2C : Amber solid 8 weeks-olded kitten with dark nose, dark paw pads and ghost tabby pattern, a/a, D/-, i/i, o/o, s/s, -/-.
48x30mm (300 x 300 DPI)

Figure 2D : Same cat as picture 2C at 9 months old: note the tabby pattern toning down to an apricot-coloured coat with dark paw pads and nose.
48x32mm (300 x 300 DPI)

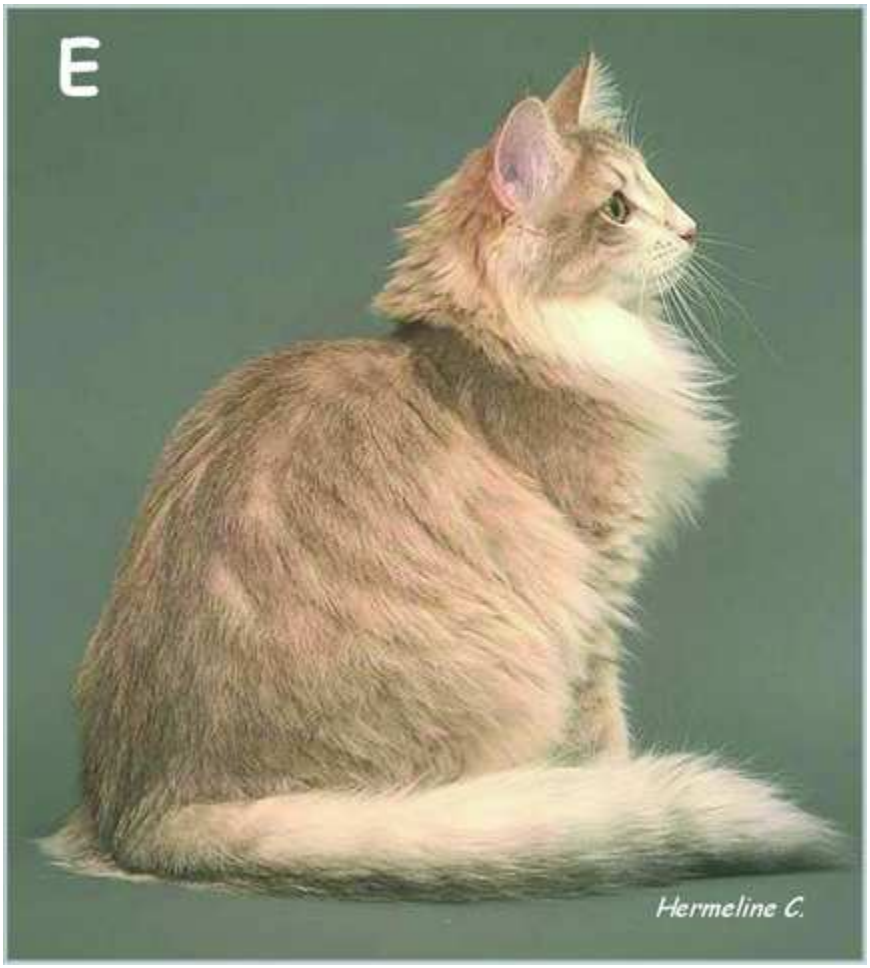

Figure 2E : Amber light silver mackerel tabby female with little white on the breast at 10 months old, A/-, d/d, I/-, o/o, S/-, Tm/-. She has a light pinkish-beige colour and her tabby pattern is already toned down.
36x40mm (300 x 300 DPI)

Figure 2F : Amber silver tortoiseshell mackerel tabby and white female at 8 weeks old, A/-, D/-, I/-, O/o, S/-, Tm/-. Differentiation between orange (e.g. left forelimb) and amber is still easy.
32x48mm (300 x 300 DPI)

Figure 2G : Same cat as Figure 2F at 12 months old: dark stripes brightened and became tawny, mistakable for orange areas (e.g. right backlimb).
48x33mm (300 x 300 DPI)

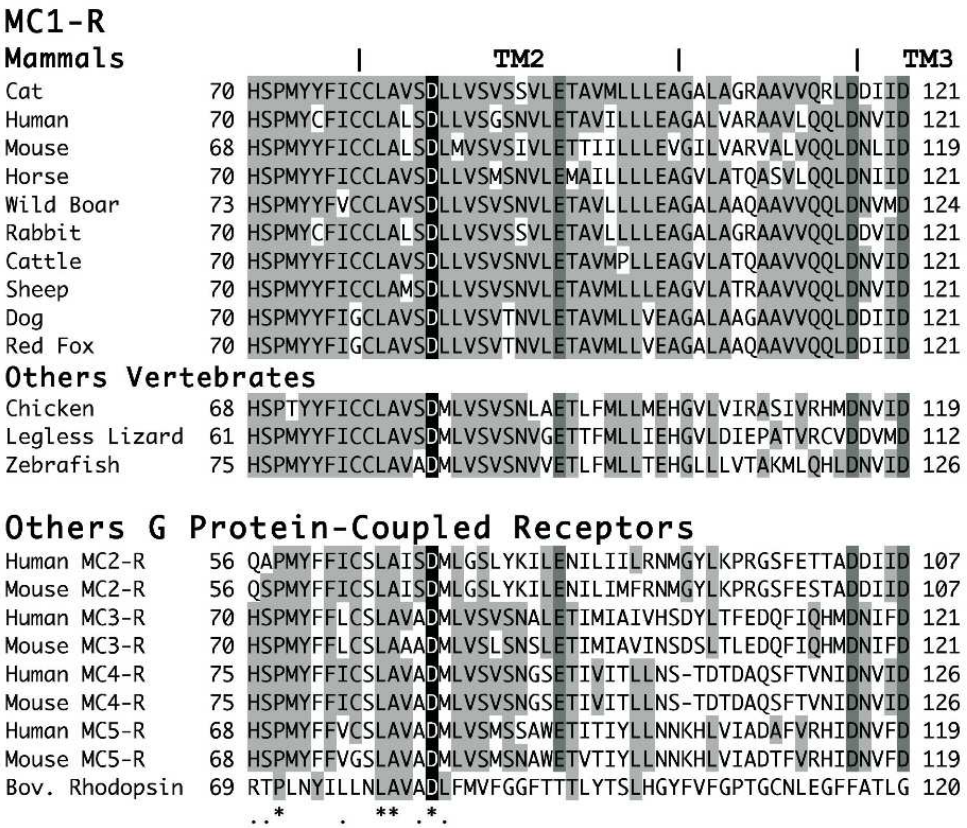

Figure 3: Alignment of the protein region encompassing the MC1-R second transmembrane segment for 22 Rhodopsin related G Protein-Coupled Receptors The multiple alignment was performed with the complete sequence by using CLUSTALW with default parameters TM2 corresponds to the second transmembrane field amino acid sequence (residues 75 to 100). TM3 corresponds to the third transmembrane field amino acid sequence (residues 114 to 144) (Ringholm et al., 2004). X, identity in most conserved residues in relation to the melanocortin 1 receptor sequences. D represents Asp84; E represents Glu94; D represents Asp117 & Asp121 according to the MC1-R feline sequence (Q865E9). Accession numbers in the protein database: MC1-R from cat (Q865E9), human (Q01726), mouse (Q01727), horse (P79166), wild boar (Q9TU05), rabbit (CAJ57384), cattle (NP_776533), sheep (CAA74298), dog (AAC33737), red fox (CAA62349), chicken (BAD91484), legless lizard (AAT90151) and zebrafish (NP_851301); MC2-R from human (Q01718) and mouse (NP_032586); MC3-R from human (NP_063941) and mouse (NP_032587); MC4-R from human (P32245) and mouse (P56450); MC5-R from human (P33032) and mouse (P41149); Bovine Rhodopsin (NP_001014890).
159x136mm (150 x 150 DPI)

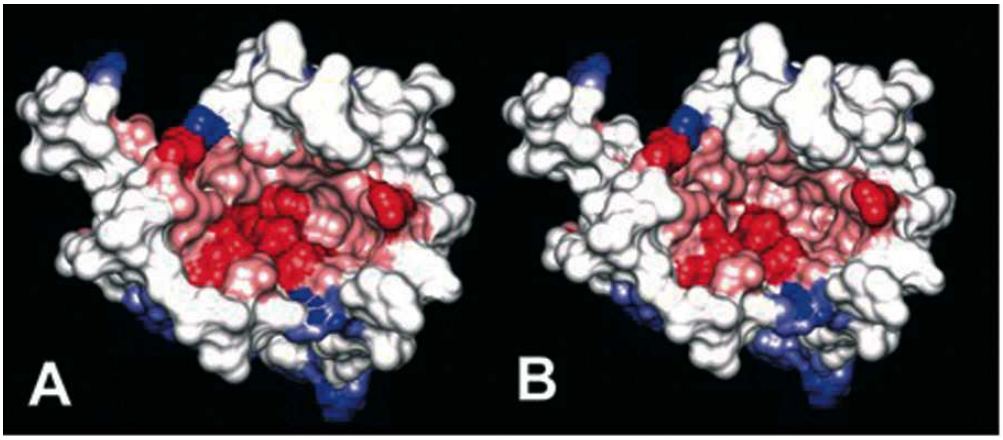

Figure 4: 3D models of wild-type and mutant melanocortin 1 receptors. The 3D models of transmembrane moities of wild-type (A) and p.Asp84Asn mutant MC1-R (B) were built with geno3D server by using the beta-2-adrenergic receptor (2rh1 PDB code) as a template. The electrostatic potential was calculated with Delphi (Rocchia et al. 2001). The molecular surface was generated by using the MSMS program (Sanner et al. 1996). The orientation is top-down regarding the ligand binding site pocket.
153x66mm (150 x 150 DPI)

Table S1: Table of breeding types presenting the number of cats produced with and without the amber phenotype.

Mating (n)	Kittens	Female		Male	
	Phenotypes	Amber	WT	Amber	WT
amber x amber (20)		44	0	45	0
WT non-carrier x amber (20)		0	54	0	48
WT carrier x amber (20)		25	20	23	23
WT carrier x WT carrier (14)		7	22	8	21
WT non-carrier x WT non-carrier (20)		0	42	0	45

WT, wild-type phenotype (non-amber colour)