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Mutation in the melanocortin 1 receptor is associated with amber colour in the Norwegian Forest Cat

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Summary

Amber (previously called X-Colour) is a yellow recessive coat colour observed in the Norwegian Forest Cat (NFC) 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 for the melanocortin 1 receptor in 12 amber, three carriers, two wild-type NFCs, one wild-type European Shorthair and two 'golden' Siberian cats and identified two single nucleotide polymorphisms (SNPs): a non-synonymous (FM180571: c.250G>A) and a synonymous (FM180571: c.840T>C) mutation. 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. Three-dimensional models were built and showed electrostatic potential modification in the mutant receptor. With these results and together with those in the scientific literature, we can conclude that amber colour in NFCs is caused by a single *MC1R* allele called *e*, which has never been documented.

Keywords 3D model, amber, melanocortin 1 receptor, mutation, Norwegian Forest Cat.

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 (Fig. 1a). Amber NFC genealogies, partially represented in Fig. 1, show that non-amber cats can father amber kittens and amber matings only give amber kittens. There is no 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), and *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 to produce a large colour variability: amber tabby (Fig. 2a,b) with the three patterns, ticked (T^a), mackerel (T^m) and blotched (t^b), or amber non-agouti (solid) with ghost tabby pattern (Fig. 2c,d). 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 (Fig. 2d), in contrast to pink-nosed amber tabby cats (Fig. 2e) with pink paw pads at birth, which darken afterwards if there are no white marks in these body regions. These observations were confirmed by testing amber NFC for the *agouti* allelic series. Amber colour also exists in dilute (*d*) (Fig. 2a,e), silver (*A*/−, *I*/−) (Fig. 2e) or smoke (*a*/*a*, *I*/−), eventually in tortoiseshell (*O*/*o*) (Fig. 2f,g), and possibly with white (*S*) (Fig. 2b). Age-dependent colour maturation is clearly surprising; all kittens are initially brown tabby or blue tabby for the dilute coat (Fig. 2a,c), and then their original colour brightens and adults show an apricot/cinnamon-like colour (Fig. 2b,d) or pinkish beige/fawn-like colour, called amber light (Fig. 2e) with a few dark hairs on the back and tail (Fig. 2b) and dark eye rims. Amber tortoiseshell female kittens present distinct black and red regions (Fig. 2f), then black hairs become apricot and red hairs remain unchanged in adults (Fig. 2g). A mating between an amber tortoiseshell

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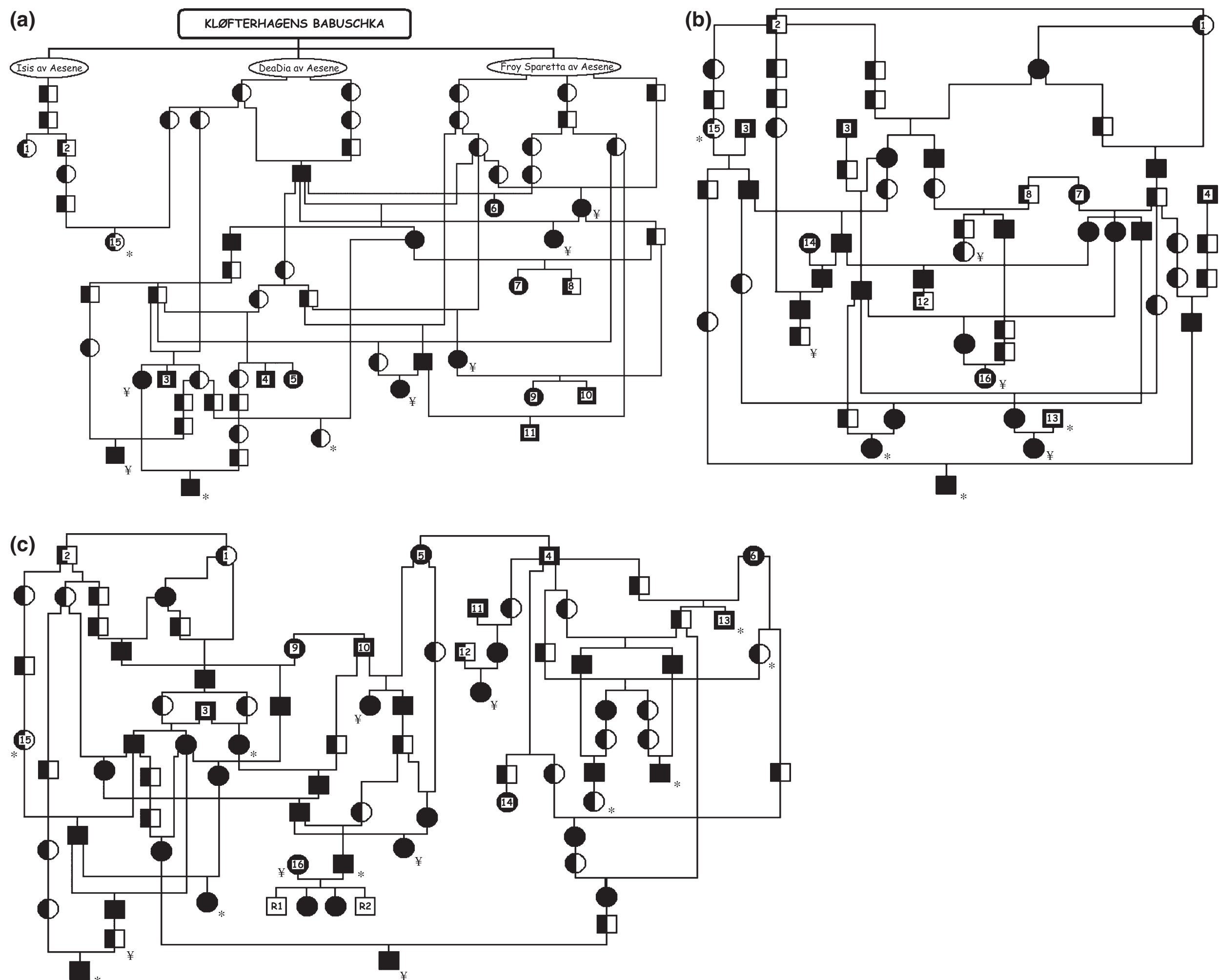

Figure 1 Pedigree analysis of amber NFC lineages. (a) Swedish lineage; (b) Dutch lineage; (c) German lineage. Circles represent females, squares represent males. Isis, DeaDia and Froy Sparetta av Aesene are daughters from the first amber carrier, Kløfterhagens Babuschka. These three daughters were very probably amber carriers. Numbers within the symbols represent the same cats in each lineage. Half-coloured symbols represent amber carrier 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. ¥cats whose MC1R region was sequenced (15 animals); *certain cats whose MC1R region was genotyped by PCR-RFLP (13 animals), other tested cats share common ancestors.

dame and an amber sire gave two amber females and two red males (see cats R1 and R2 in Fig. 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 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 because of 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

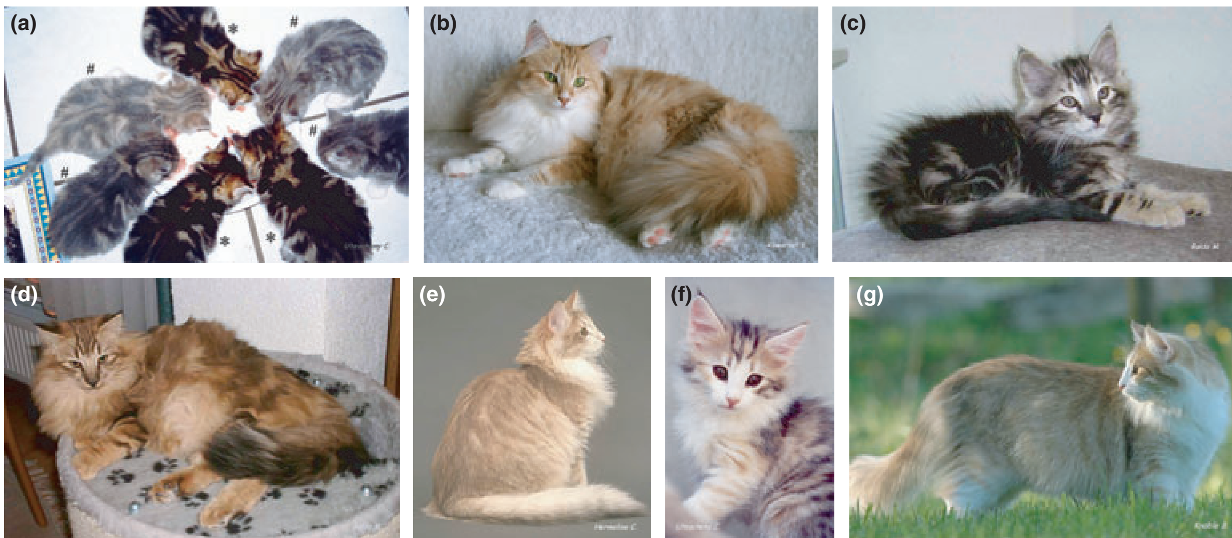

Figure 2 Photographs and selected genotypes illustrating the variety of amber colours in the NFCs. (a) A 4-week-old litter of three amber blotched tabby (*), *A/−*, *D/−*, *i/i*, *O/o*, *s/s*, t^b/t^b and four light amber blotched tabby kittens (#), *A/−*, *d/d*, *i/i*, *O/o*, *s/s*, t^b/t^b. Areas between 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 (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 figure (f) at 12 months old: dark stripes brightened and became tawny, mistakable for orange areas (e.g. right back limb).

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 three wild-type cats (two NFC and one European Shorthair), 33 amber NFC, 36 carrier NFC and four '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 (954 bp) in 12 amber, three carriers, two wild-type NFCs, one wild-type European Shorthair and two 'golden' Siberian cats after PCR amplification. The MC1R gene sequencing displayed in all sequences the same silent 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 two 'golden' Siberian cats) by RFLP-PCR using BstXI (Fermentas) and Hpy188I (New England Biolabs), which cleave the c.250A and the c.250G alleles respectively. Primers forward (5′-TGCTGGGCTCCCTCAACTC-3′) and reverse (5′-CCAG CACGTCAATGATGTCG-3′) were designed to amplify a 342-bp fragment (29–370). 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 coding gene from 43 cats of various breeds. 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 (Fig. 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 (Fig. 3). The human p.Asp84Glu variant was reported in several MC1R coding region sequencing studies but with discrepant findings, because it was not always significantly associated with

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MC1-R
Mammals                            |         TM2         |            |  TM3
Cat             70 HSPMYYFICCLAVSDLLVSVSSVLETAVMLLLEAGALAGRAAVVQRLDDIID 121
Human           70 HSPMYCFICCLALSDLLVSGSNVLETAVILLLEAGALVARAAVLQQLDNVID 121
Mouse           68 HSPMYYFICCLALSDLMVSVSIVLETTIILLLEVGILVARVALVQQLDNLID 119
Horse           70 HSPMYYFICCLAVSDLLVSMSNVLEMAILLLLEAGVLATQASVLQQLDNIID 121
Wild Boar       73 HSPMYYFVCCLAVSDLLVSVSNVLETAVLLLLEAGALAAQAAVVQQLDNVMD 124
Rabbit          70 HSPMYCFICCLALSDLLVSVSSVLETAVLLLLEAGALAGRAAVVQQLDDVID 121
Cattle          70 HSPMYYFICCLAVSDLLVSVSNVLETAVMPLLEAGVLATQAAVVQQLDNVID 121
Sheep           70 HSPMYYFICCLAMSDLLVSVSNVLETAVMLLLEAGVLATRAAVVQQLDNVID 121
Dog             70 HSPMYYFIGCLAVSDLLVSVTNVLETAVMLLVEAGALAAGAAVVQQLDDIID 121
Red Fox         70 HSPMYYFIGCLAVSDLLVSVTNVLETAVMLLVEAGALAAQAAVVQQLDDIID 121
Others Vertebrates
Chicken         68 HSPTYYFICCLAVSDMLVSVSNLAETLFMLLMEHGVLVIRASIVRHMDNVID 119
Legless Lizard  61 HSPMYYFICCLAVSDMLVSVSNVGETTFMLLIEHGVLDIEPATVRCVDDVMD 112
Zebrafish       75 HSPMYYFICCLAVADMLVSVSNVVETLFMLLTEHGLLLVTAKMLQHLDNVID 126

Others G Protein-Coupled Receptors
Human MC2-R     56 QAPMYFFICSLAISDMLGSLYKILENILIILRNMGYLKPRGSFETTADDIID 107
Mouse MC2-R     56 QSPMYFFICSLAISDMLGSLYKILENILIMFRNMGYLKPRGSFESTADDIID 107
Human MC3-R     70 HSPMYFFLCSLAVADMLVSVSNALETIMIAIVHSDYLTFEDQFIQHMDNIFD 121
Mouse MC3-R     70 HSPMYFFLCSLAAADMLVSLSNSLETIMIAVINSDSLTLEDQFIQHMDNIFD 121
Human MC4-R     75 HSPMYFFICSLAVADMLVSVSNGSETIVITLLNS-TDTDAQSFTVNIDNVID 126
Mouse MC4-R     75 HSPMYFFICSLAVADMLVSVSNGSETIVITLLNS-TDTDAQSFTVNIDNVID 126
Human MC5-R     68 HSPMYFFVCSLAVADMLVSMSSAWETITIYLLNNKHLVIADAFVRHIDNVFD 119
Mouse MC5-R     68 HSPMYFFVGSLAVADMLVSMSNAWETVTIYLLNNKHLVIADTFVRHIDNVFD 119
Bov. Rhodopsin  69 RTPLNYILLNLAVADLFMVFGGFTTTLYTSLHGYFVFGPTGCNLEGFFATLG 120
                   ..*     .  ** .*.
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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 75–100). TM3 corresponds to the third transmembrane field amino acid sequence (residues 114 to 144) (Ringholm *et al.* 2004). Light grey highlighted amino acids are conserved residues in relation to the melanocortin 1 receptor sequences. Dark highlighted aspartate (D) represents Asp84; dark grey-highlighted amino acids represent respectively Glu94, Asp117 and 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).

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 is associated with 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 (Fig. 3). To check the impact of the p.Asp84Asn mutation, 3D models were built on the Geno3D server (Combet *et al.* 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 (Fig. 4a) and the mutant (Fig. 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 to the side chain 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 interacts with the arginine 8 from the α-MSH (Yang *et al.* 1997). These three acidic residues (Glu94, Asp117 and

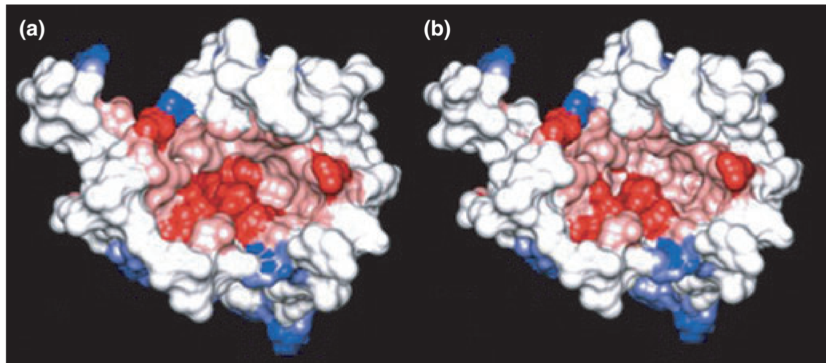

Figure 4 3D models of wild-type and mutant melanocortin 1 receptors, The 3D models of transmembrane moities of wild-type (a) and p.Asp84Asn mutant MC1-R (b) were built with geno3D server by using the beta-2-adrenergic receptor (2rh1 PDB code) as a template. The electrostatic potential was calculated 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.

Asp121) are well conserved among the melanocortin receptor amino acid sequences from all species (Fig. 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 with *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 three 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, Fig. 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 the production of a large range of yellow colours, from tawny (Fig. 2g) to red-apricot (Fig. 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).

Epistatic effects from the inactivating recessive mutation *e* were first reported in mice (Robbins *et al.* 1993), but seem to be quite different than the *e* mutation in cats, because this epistasy is not observed in amber kittens. Adult amber coats are apricot and the tabby pattern is very faint regardless of 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 (Fig. 2a) with a black tip, whereas non-agouti hairs are initially black and become apricot afterwards (Fig. 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 (Fig. 2d).

Finally, we also studied MC1R coding gene in four '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 may share common ancestors. The Siberian MC1R coding region 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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Supporting information

Additional supporting information may be found in the online version of this article:

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

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