

Thr40 and Met122 are new partial loss-of-function natural mutations of the human melanocortin 1 receptor

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Abstract Activation by melanocortins of the melanocortin 1 receptor (MC1R), expressed in epidermal melanocytes, stimulates melanogenesis. Human *MC1R* gene loss-of-function mutations are associated with fair skin, poor tanning and increased skin cancer risk. We identified two natural alleles: Ile40Thr, probably associated with skin types I–II, and Val122Met. Val122Met bound [¹²⁵I][Nle⁴, D-Phe⁷]- α -melanocyte stimulating hormone with lower affinity than the wild-type. Dose–response curves of cAMP accumulation were right-shifted for both forms. The Val122Met form failed to achieve maximal cAMP responses comparable to the wild-type or Ile40Thr receptors. Thus, the Ile40Thr and Val122Met variants are partial loss-of-function natural mutations of *MC1R*. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Melanocortin 1 receptor; Melanogenesis; Melanocyte; Skin type; G protein-coupled receptor; cAMP

1. Introduction

The melanocortin receptors (MCR) are a well defined subgroup of the G protein-coupled receptors (GPCR) family, showing significant sequence similarity, from 40 to 60%. Compared to other GPCRs, the MCRs are small (298–372 amino acids), with short external amino- and internal carboxyl-termini. The second extracellular and fifth transmembrane (TM) domains are also unusually short (reviewed in [1]). Their ligands derive from the precursor proopiomelanocortin, which undergoes a complex and tissue-specific posttranslational processing, leading to ACTH, α -, β -, and γ -melanocyte stimulating hormone (MSH), collectively termed melanocortins, and other bioactive peptides [2].

MC1R is the only MCR expressed in melanocytes [1], the epidermal cells responsible for mammalian pigmentation. It displays the typical characteristics of the GPCR family [3], with seven TM fragments. In the mouse, its preferential ligand is α -MSH, but the human receptor is equally responsive to ACTH. Upon binding to MC1R in the melanocyte plasma

membrane, these hormones trigger the cAMP cascade and activate melanogenesis [4]. Therefore α -MSH and ACTH are critical for the regulation of skin pigmentation.

In the mouse, the gene encoding the 315 amino acids Mc1r maps to the extension locus [5]. There are four naturally occurring extension alleles: wild-type (E^+), sombre (E^{so} and E^{so-3J}), tobacco (E^{to}), and recessive yellow (e). The gain-of-function dominant E^{so} allele is associated with black pigmentation and overexpression of the darker pigments known as eumelanins, with respect to the lighter, yellowish to reddish, pheomelanins. Another gain-of-function allele, E^{to} , also causes dominant black pigmentation. Conversely, mice homozygote for the loss-of-function e allele lack eumelanin and are almost entirely yellow [5]. Therefore, a clear relationship exists between Mc1r function, melanin type and fur color.

The human MC1R is a 317 amino acid protein. The *MC1R* gene was independently cloned in 1992 by two groups, who reported slightly different sequences [6,7]. Some of the differences might be sequencing errors [8], although others correspond to real polymorphisms. Indeed, the *MC1R* gene is highly polymorphic [9,10]. Human skin types are usually classified into different groups, depending on their basal pigmentation and sensitivity to UV solar radiation. The frequently used modified Fitzpatrick's scale for Caucasian skin considers four different types, with increasing basal pigmentation and UV-induced tanning response, from type I (fair skin, burns easily, never tans), to type IV (dark skin, tans well, rarely burns). Based on the key role of mouse *Mc1r* on pigmentation in mice, an association of human *MC1R* variants and skin type has been actively investigated. The best studied variants are Val60Leu, Arg67Gln, Asp84Glu, Val92Met, Arg121His, Arg142His, Arg151Cys, Ile155Thr, Arg160Trp, Arg163Gln, Asp294His, all resulting from point mutations, and several insertions as 86insA, and 537insC [11–17]. Some variants are more frequent in individuals with sun-sensitive skin types I or II than in persons with higher basal pigmentation and tanning response [15–17]. In addition, their frequency is higher in blonds or red heads than in black-haired subjects, thus also relating hair color and *MC1R* [11,13,17]. The functional effects of these variants have been analyzed. Arg142His, Arg151Cys, Arg160Trp and Asp294His are loss-of-function alleles [14]. The situation is less clear for the Val60Leu, since transfection studies strongly suggest that it may be a partial loss-of-function mutation [14], but, on the other hand, association studies did not find any significant correlation with a particular skin or hair phenotype [13,15,17]. In any case, *MC1R* seems a major determinant of human skin type and

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Abbreviations: AS-PCR, allele-specific polymerase chain reaction; GPCR, G protein-coupled receptor; MC1R, melanocortin 1 receptor; α -MSH, α -melanocyte stimulating hormone; NDP-MSH, [Nle⁴, D-Phe⁷]- α -melanocyte stimulating hormone; TM, transmembrane

hair color, and an impairment of receptor function appears associated with fair skin type or red hair. Since pigmentation and skin cancer are also related, *MC1R* is considered a genetic factor of skin cancer risk [13,15,18,19].

In a study of *MC1R* allelic variants as related to skin type and melanoma risk we identified two new variants in skin type I or II individuals: Ile40Thr and Val122Met (GenBank accession numbers AY046528 and AY046529, respectively). These variants were further studied since they are located in regions potentially important for receptor function (first and third TM fragments [3]). A schematic representation of the structure of *MC1R* can also be found on our Web page (<http://www.um.es/bmbi/melanocitos.htm>). We show here that these variants lead to impaired binding affinity of *MC1R* for its cognate ligands, and lower efficiency of coupling to the cAMP cascade.

2. Materials and methods

2.1. DNA extraction, amplification and cloning of *MC1R*

Genomic DNA was extracted from blood samples of healthy volunteers, using the Wizard kit (Promega, Madison, WI, USA). The entire coding sequence of the *MC1R* gene was amplified using primers CCTAAGCTTACTCCTTCTGCTTCCTGGACA (forward, matching nucleotides 435–456) and CTGGAATTCACACTTAAAGCGCGTGCACCGC (reverse, matching nucleotides 1418–1439), designed from the sequence reported in [6], GenBank database entry X65634, containing added *Hind*III and *Eco*RI restriction sites (underlined). The 1023 bp fragment was cloned in pcDNA3 (Invitrogen, Carlsbad, CA, USA) by means of the added restriction sites. PCR was performed using 0.5 µg of target DNA, 0.35 µg of each primer, 200 µM each dNTP and 2.5 U of the proofreading *Pfu* polymerase. Thirty rounds of amplification were carried out with denaturation for 1 min at 95°C, annealing for 2 min at 68°C and extension for 3 min at 72°C, with a final 10 min extension at 72°C. Complete sequencing of two PCR products, obtained in independent reactions, and of the cloned inserts was performed in both strands.

2.2. Allele-specific PCR (AS-PCR)

The forward primers CGGTGCCTGGAGGTGCCAC (Fw-Thr40) and CAGCTGGACAATGTCATTGACA (Fw-Met122), where the 3'-terminal nucleotide responsible for specificity is shown in bold, were designed for the specific detection of the Ile40Thr and Val122Met alleles. Both primers were used with the reverse primer described above. PCR conditions were: 30 rounds of 1 min at 95°C for denaturing, 1 min at 68 or 64°C for annealing of Fw-Thr40 and Fw-Met122, respectively, and 90 s at 72°C for extension, with a final extension step of 10 min at 72°C.

Table 1
Frequency of the Ile40Thr, Val60Leu, Val92Met, and Val122Met *MC1R* allelic variants

Variant	Frequency (% carriers)		Hair color				Overall (n = 55)
	Skin type		Overall (n = 55)				
	Types I–II (n = 28)	Types III–IV (n = 27)	Fair (n = 25)	Light brown (n = 8)	Dark brown and black (n = 19)	Red (n = 3)	
Ile40Thr	14.3 (4)	0 (0)	16 (4)	0 (0)	0 (0)	0 (0)	7.3 (4)
Val60Leu	17.8 (5)	11.1 (3)	24 (6)	0 (0)	10.5 (2)	0 (0)	14.5 (8)
Val92Met	10.7 (3)	3.7 (1)	12 (3)	0 (0)	5.3 (1)	0 (0)	7.3 (4)
Val122Met	14.3 (4)	7.4 (2)	12 (3)	0 (0)	10.5 (2)	33 (1)	10.9 (6)

A total of 55 healthy volunteers were classified into two groups according to skin type (types I–II and types III–IV), and into four groups according to hair color. For convenience, and to avoid classification errors, no effort was made to distinguish between skin types I or II, on one hand, and III or IV, on the other. DNA was extracted from 3 ml blood samples. All AS-PCR reactions were performed with 0.5 µg of target DNA, as described in Section 2. The complete coding sequence of the *MC1R* gene from samples found positive for any one of the mutations was amplified and sequenced for confirmation and identification of homozygote carriers. All carriers were heterozygous for the mutations under study, carrying one mutant and one wild-type allele, except for one Val60Leu homozygote (skin type I–II, blond hair). Two other individuals were compound heterozygotes, one for the Ile40Thr and Val60Leu variants and the other for the Val60Leu and Arg163Gln alleles, and both of them had skin type I–II and blond hair.

n, number of individuals in each group. Allele frequencies are shown as percentage of individuals carrying at least one mutant allele, whose number is shown in parentheses, with respect to the total population studied in each column.

2.3. Transient expression of *MC1R*

HEK 293T cells were grown in six-well plates with RPMI 1640, 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. This cell line has been extensively used in previous studies of transfected *MC1R* genes, since it does not express endogenous α -MSH binding sites [6,8]. Transfection was carried out with the Superfect reagent (Qiagen, Paisley, UK), as per instructions, with 1.5 µg plasmid DNA/well. Three hours after adding the transfection mix, the medium was removed. Cells were gently washed with 500 µl phosphate-buffered saline (PBS) and 1 ml of fresh medium was added. After 24 h, cells were completely serum-deprived for an additional 24 h before binding or coupling assays.

2.4. Radioligand binding assay

Transfected cells were incubated (1 h, 37°C) with increasing concentrations of [¹²⁵I]- α -MSH (NDP-MSH, Sigma, Saint Louis, MO, USA), ranging from 10⁻¹² to 10⁻⁶ M and a fixed amount of [¹²⁵I]NDP-MSH (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK), corresponding to 10⁻¹⁰ M and 0.1 µCi/well, in a final volume of 500 µl RPMI. Cells were then washed twice with RPMI for 5 min at room temperature, and trypsinized. The cell suspension was pipetted into plastic tubes and the associated radioactivity was measured. Non-specific binding was estimated from the radioactivity bound in the presence of 10⁻⁶ M NDP-MSH or to cells transfected with empty pcDNA and incubated with [¹²⁵I]NDP-MSH alone, with similar results.

2.5. Determination of agonist-induced cAMP increases

Cells grown as above and serum-deprived for 24 h were incubated with 10⁻¹³–10⁻⁷ M NDP-MSH, for 20 min. Then, the medium was aspirated, cells quickly washed with 1 ml ice-cold PBS, lysed with 350 µl preheated 0.1 N HCl (70°C), and carefully scraped. The resulting mix was freeze-dried for 2 h, washed with 100 µl H₂O, and freeze-dried for another 30 min. Dried samples were dissolved in suitable volumes of 50 mM Tris, 4 mM EDTA, pH 7.5, from 65 to 300 µl, depending on the expected cAMP content. cAMP was measured by radioimmunoassay (Amersham Pharmacia Biotech), as per instructions. Parallel dishes for protein determination were included. Cells were dissolved in 10 mM phosphate buffer pH 7, 1% Igepal CA-640, containing 0.1 mM EDTA, and 0.1 mM PMSF. The protein concentration in these lysates was determined by the bicinchoninic acid method.

3. Results and discussion

Sequencing of the complete *MC1R* open reading frame from a small sample of five skin type I or II individuals, and five others with skin type III or IV confirmed that *MC1R* allelic variants are common in fair skin subjects, but not in individuals with darker skin and high tanning ability.

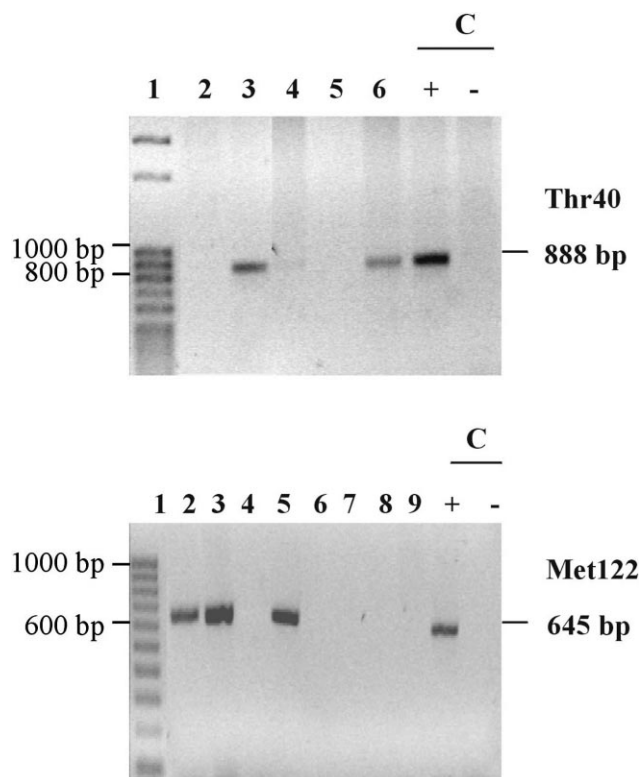


Fig. 1. AS-PCR analysis of the Ile40Thr and Val122Met variants in human genomic DNA. The size of the expected PCR fragments was 888 bp for the Ile40Thr allele (upper panel) and 645 bp for the Val122Met variant (lower panel). Lane 1 in each panel shows the migration of molecular size standards. The following lanes correspond to a representative number of samples from different individuals. In the two last lanes on the right, C stands for controls. These were ascertained by complete sequencing of the *MC1R* gene, and were included in each AS-PCR round. The positive control (+) consisted of an identical amount of genomic DNA from a heterozygote harboring one variant and one wild-type allele. The negative control (–) corresponded to a wild-type homozygote. Aliquots (1/5 of the PCR reaction volume) were electrophoresed in 2% agarose gels and ethidium bromide-stained. The negative of the stained gel is shown, for easier visibility.

Whereas all five skin III–IV individuals were homozygous for wild-type *MC1R* (GenBank accession number AF326275), four skin I–II volunteers were heterozygotes harboring Val92Met (2/5), Ile40Thr (1/5) and Val122Met (1/5) alleles, and the fifth individual was a compound heterozygote for the Val60Leu and Arg163Gln alleles. These data are in agreement with previous reports of a high frequency of *MC1R* variants in skin I and II individuals [15–17]. Among these variants, Val60Leu, Val92Met, and Arg163Gln have already been described. The Arg163Gln variant is highly frequent in Asian population and

does not seem to have any relevant functional effect [9,11]. Therefore, it was not included in our study. The Val60Leu allele encodes a partial loss-of-function protein, with binding properties almost identical to the wild-type, but a residual signaling through the cAMP cascade [14], and no clear association with any particular skin type or hair color [13,15,17]. Concerning the Val92Met substitution, a study reported a lower affinity for α -MSH [20], whereas others did not find significant functional changes [21]. In any case, this suggests that the functional effects of the Val92Met substitution, if any, are small, a conclusion strengthened by association studies [17].

However, variants Ile40Thr and Val122Met have not been found in previous studies involving a much larger number of individuals. Therefore, we wished for confirmation of their existence and for an estimation of their frequency, by performing an AS-PCR analysis of a larger group. For comparison, the well characterized Val60Leu and Val92Met alleles were also analyzed. AS-PCR confirmed the sequencing data (Fig. 1). The frequency data for the Val60Leu and Val92Met alleles were in reasonable agreement with those reported by others [17], thus validating the screening technique. Variants Ile40Thr and Val122Met were more frequently found in skin type I and II individuals (Table 1). This was particularly clear for the Ile40Thr allele, that was found exclusively in skin type I–II subjects, thus suggesting a possible association with fair skin. However, the size of the population analyzed was relatively small, and the *P* values obtained from the Fisher's exact test only approached significance for the Ile40Thr variant (*P* = 0.06). Work is underway to increase the number of samples analyzed and confirm or disprove a possible association of this allele with fair skin and/or blond hair. In any case, the Ile40Thr and Val122Met alleles are relatively frequent, at least in the Spanish population.

According to current models of the *MC1R* protein [3], Ile40 lies in the extracellular side of the first TM fragment (TM1), and Val122 is located in TM3, next to Asp121, a residue found to be critical for agonist binding by site-directed mutagenesis studies [22]. TM1 and TM3 contain important determinants for binding of agonists and effective coupling to the cAMP cascade [1]. Moreover, Ile40 and Val122 are highly conserved residues, present in most mammalian *MC1R* genes sequenced to date [1,3]. Ile40 is also present in the mouse and human MC3 and MC5 receptors [3]. Therefore, we analyzed the functional effects of the Ile40Thr and Val122Met substitutions.

The binding affinity of the variants was studied by competition binding experiments performed with HEK 293T cells transiently expressing the receptor protein (Fig. 2). Displacement curves were significantly shifted to higher competing ligand concentrations, as compared to the wild-type. IC_{50} val-

Table 2
Binding and coupling parameters of wild-type *MC1R* and the Ile40Thr and Val122Met variants transiently expressed in HEK 293 cells

Variant	B_{max} (fmol/mg protein)	IC_{50} for displacement by NDP-MSH (nM)	EC_{50} for cAMP production (nM)	Maximal cAMP levels (pmol/mg protein)
Wild-type	3270 ± 610	0.70 ± 0.12	0.011 ± 0.004	355 ± 60
Ile40Thr	4880 ± 870	2.66 ± 0.41**	1.10 ± 0.23**	326 ± 27
Val122Met	8440 ± 540	16.41 ± 3.4**	0.36 ± 0.09**	185 ± 56*

All data are given as mean ± S.E.M. ($n \geq 4$). The statistical significance of the differences in IC_{50} , EC_{50} and maximal cAMP levels was assessed by a one-tailed Student's *t*-test.

**P* < 0.05.

***P* < 0.005.

ues for [125 I]NDP-MSH displacement by unlabeled NDP-MSH were 7.0×10^{-10} M for the wild-type, 2.7×10^{-9} M for Ile40Thr and 1.6×10^{-8} M for Val122Met (Table 2). Therefore, the Val122Met allele results in a clear reduction in affinity for NDP-MSH, whereas this effect is lower, although statistically significant, for the Ile40Thr form. An effect on affinity for agonists is not surprising for the Val122Met substitution, since Asp121 is a major determinant of hormone binding [3,22], so that a mutation close to this residue, which is thought to interact with the hormone, can easily impair ligand docking. The loss of affinity of the Ile40Thr variant is less marked, but still evident. Interestingly, computer modeling of the MC1R three-dimensional structure suggests that a cavity formed between TM1 and TM3 is important for binding to the melanocortins core sequence. Within this cavity, residues Leu48, Ser52 and Glu55 in TM1 are thought to interact with the ligand, and all these residues are located in the same side of the predicted helical structure as Ile40 [3].

The functional coupling of the Ile40Thr and Val122Met variants was then analyzed. HEK 293T cells were transiently transfected with the wild-type, Ile40Thr and Val122Met forms, and their cAMP levels in response to increasing doses of NDP-MSH were measured (Fig. 3). Both mutant receptors displayed a rightward shift in the dose–response curves, with significant changes in the EC_{50} for agonist-induced cAMP accumulation (1.1×10^{-11} M for wild-type, 1.1×10^{-9} M for Ile40Thr and 3.6×10^{-10} M for Val122Met). Moreover, the basal and maximal hormone-induced cAMP levels were similar for wild-type and Ile40Thr receptors, but significantly lower for the Val122Met mutant (Fig. 3 and Table 2).

A comparison of the shifts in IC_{50} for agonist displacement and EC_{50} for cAMP production, together with the maximal levels of cAMP achieved after stimulation with saturating doses of NDP-MSH, prove that the functional effects of the two mutations are different. The affinity of Ile40Thr MC1R for NDP-MSH is decreased less than four-fold, as compared to the wild-type. Conversely, the EC_{50} for coupling to the cAMP-generating system is increased about 100-fold. There-

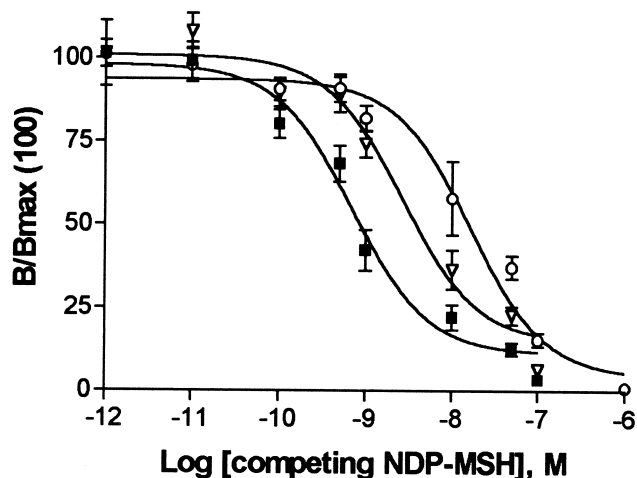


Fig. 2. Binding of NDP-MSH to wild-type MC1R and the Ile40Thr and Val122Met variants. Measurements were performed in quadruplicate independent dishes. The results were fitted to a one site competition binding curve, using GraphPad Prism 2.01 (GraphPad Software, San Diego, CA, USA). Error bars indicate S.E.M. ■, wild-type; ▽, Ile40Thr; ○, Val122Met.

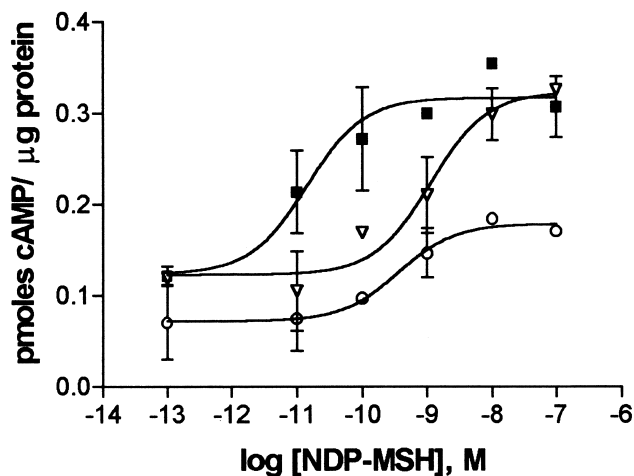


Fig. 3. Functional coupling of the Ile40Thr and Val122Met variants. HEK 293T cells transfected with the wild-type and mutant forms, and treated with 10^{-13} – 10^{-6} M NDP-MSH, for 20 min, prior to lysis and determination of their cAMP contents. Each measurement was performed in duplicate or triplicate independent wells, and the complete set of measurements was repeated at least twice ($n \geq 4$). Results were fitted to a sigmoidal dose–response curve. Error bars indicate S.E.M. ■, wild-type; ▽, Ile40Thr; ○, Val122Met.

fore, much higher fractional occupancies of MC1R seem necessary for the Ile40Thr variant to achieve similar cAMP responses as the wild-type receptor. These data strongly suggest that the Ile40Thr substitution not only impairs hormone binding, but also decreases the ability of the hormone–receptor complex to undergo the conformational change leading to receptor activation and efficient coupling to G_s . Conversely, the affinity of the Val122Met for NDP-MSH, and the EC_{50} value for coupling to cAMP production are affected to approximately the same extent. This suggests that the rightward shift in the cAMP dose–response curve for this mutant is accounted for by decreased binding affinity. But, in this case, the maximal levels of cAMP elicited by saturating doses of the agonist are lower than for the wild-type. Therefore, the final active conformational state of the Val122Met mutant seems less efficient in G_s activation, in a way reminiscent of the situation observed after binding of partial agonists to other GPCRs.

Natural MC1R mutations leading to impaired coupling without noticeable effects on agonist binding affinity have been described [14]. This, together with the results presented here, shows that natural loss-of-function mutations impairing virtually all the steps of MC1R activation are actually present. Their study may help to reconcile occasional discrepancies between data obtained by functional approaches in transfected heterologous cells on one hand, and by analysis of the association of particular variants with skin or hair pigmentation phenotypes, on the other. The functional analysis of natural MC1R variants should therefore allow not only for a better understanding of the genetic determinants of skin type, hair color and UV response [23], but also for a dissection at the molecular level of the mechanisms of MC1R activation. Interestingly, and quite surprisingly in the light of the frequent loss-of-function mutations and of the high human MC1R gene polymorphism, no activatory mutation such as the ones described for the mouse gene has been described so far.

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