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Zhi-Qiang Wang, Ya-Xiong Tao. Functional Studies on Twenty Novel Naturally Occurring Melanocortin-4 Receptor Mutations. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2011, 1812 (9), pp.1190. 10.1016/j.bbadis.2011.06.008 . hal-00719827

**HAL Id: hal-00719827**

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Submitted on 21 Jul 2012

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## Accepted Manuscript

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PII: S0925-4439(11)00134-7  
DOI: doi: [10.1016/j.bbadis.2011.06.008](https://doi.org/10.1016/j.bbadis.2011.06.008)  
Reference: BBADIS 63304

To appear in: *BBA - Molecular Basis of Disease*

Received date: 21 April 2011  
Revised date: 8 June 2011  
Accepted date: 21 June 2011



Please cite this article as: Zhi-Qiang Wang, Ya-Xiong Tao, Functional Studies on Twenty Novel Naturally Occurring Melanocortin-4 Receptor Mutations, *BBA - Molecular Basis of Disease* (2011), doi: [10.1016/j.bbadis.2011.06.008](https://doi.org/10.1016/j.bbadis.2011.06.008)

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**Functional Studies on Twenty Novel Naturally Occurring Melanocortin-4 Receptor Mutations**

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Running title: Functions of novel melanocortin-4 receptor mutations

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**Abstract**

The melanocortin-4 receptor (MC4R) is a G protein-coupled receptor critically involved in regulating energy balance. MC4R activation results in decreased food intake and increased energy expenditure. Genetic and pharmacological studies demonstrated that the MC4R regulation of energy balance is conserved from fish to mammals. In humans, more than 150 naturally occurring mutations in the *MC4R* gene have been identified. Functional study of mutant MC4Rs is an important component in proving the causal link between *MC4R* mutation and obesity as well as the basis of personalized medicine. In this article, we studied 20 *MC4R* mutations that were either not characterized or not fully characterized. We showed that 11 mutants had decreased or absent cell surface expression. D126Y was defective in ligand binding. Three mutants were constitutively active but had decreased cell surface expression. Eleven mutants had decreased basal signaling, with two mutants defective only in this parameter, suggesting that impaired basal signaling might also be a cause of obesity. Five mutants had normal functions. In summary, we provided detailed functional data for further studies on identifying therapeutic approaches for personalized medicine to treat patients harboring these mutations.

**Key words:** Melanocortin-4 receptor, naturally occurring mutation, intracellular retention, binding, signaling

**Abbreviations footnote:** AgRP, agouti-related protein; GPCR, G protein-coupled receptor; MC3R, melanocortin-3 receptor; MC4R, melanocortin-4 receptor; MSH, melanocyte stimulating hormone; WT, wild type.

## 1. Introduction

The melanocortin receptors are members of Family A G protein-coupled receptors (GPCRs) involved in regulating diverse functions, including pigmentation, adrenal steroid secretion, immune modulation, energy homeostasis, exocrine gland secretion, pain perception, among others. The ligands for these receptors are small peptides derived from post-translational processing of pro-opiomelanocortin, including adrenocorticotropin and  $\alpha$ -melanocyte stimulating hormone (MSH),  $\beta$ -MSH, and  $\gamma$ -MSH, as well as several other peptides [1, 2]. These receptors are unique among GPCRs in having endogenous antagonists, Agouti and Agouti-related protein (AgRP). Agouti is the antagonist for the melanocortin-1 receptor whereas AgRP is the endogenous antagonists of the melanocortin-3 and -4 receptors (MC3R and MC4R, respectively).

The MC4R is widely expressed in the central nervous system, including the hypothalamus [3]. It is a critical regulator of energy homeostasis. MC4R activation leads to decreased food intake and increased energy expenditure. When the MC4R is inactivated through gene targeting, the knockout mice have severe obesity, together with hyperglycemia and hyperinsulinemia [4]. Mice lacking *proopiomelanocortin* gene [5] or over-expressing AgRP [6, 7] are obese. Pharmacological studies confirmed the critical role of MC4R in regulating energy homeostasis (reviewed in [2, 8]).

Following these rodent studies, human genetic studies confirmed that the MC4R is also indispensable for human energy homeostasis. Two groups independently identified frameshift

mutations in the *MC4R* in patients with early-onset severe obesity in 1998 [9, 10]. It is now well accepted that mutations in the *MC4R* gene are the most common monogenic form of obesity [11], with more than 150 distinct mutations identified so far [2, 12].

Extensive functional studies were performed on some of these mutations (reviewed in [2, 12]). These studies were important in establishing whether a particular mutation is indeed pathogenic. It was suggested that three lines of evidences, including cosegregation in pedigrees, absence in nonobese controls of the same ethnicity, and impairment in receptor function, are needed to conclude that a particular *MC4R* mutation is causative of obesity [13]. These studies identified multiple functional defects. Based on these results, we proposed a classification scheme [14, 15] as follows: Class I mutants are null mutants with diminished receptor proteins; Class II mutants are retained intracellularly; Class III mutants are defective in ligand binding; Class IV mutants are defective in signaling; and finally, Class V mutants have normal function in the parameters measured.

In the current study, we performed detailed functional studies on 18 *MC4R* mutations reported recently, including I69R [16], H76R [17], M79I [16], S94N [17], D126Y [17], D146N [17], Del170 [17], I186V [18], I195S [16], F201L [17], G231V [17], P260Q [17], F280L [18], I289L [17], R305S [17], Q307X [17], Y332C [17], and Y332H [17]. They were either not characterized or characterized incompletely. I194T and L300P were identified in mouse obesity models [19] and introduced into human *MC4R* in the current study for comparison. These mutations are shown schematically in Fig. 1.

## 2. Materials and methods

### 2.1. Hormones and supplies

[<sup>125</sup>I]-NDP-MSH ([Nle<sup>4</sup>,D-Phe<sup>7</sup>]- $\alpha$ -melanocyte stimulating hormone) was obtained from the Peptide Radioiodination Service Center at the University of Mississippi (University, MS) or American Radiolabeled Chemicals (St. Louis, MO) iodinated by Dr. Robert C. Speth.  $\alpha$ -MSH and  $\beta$ -MSH were synthesized by Pi Proteomics (Huntsville, AL) and CHI Scientific (Maynard, MA), respectively, and NDP-MSH was purchased from Peptides International (Louisville, KY). Tissue culture plastic wares and cell culture media, newborn calf serum, and reagents were obtained from Corning (Corning, NY) and Invitrogen (Carlsbad, CA), respectively.

### 2.2. *In vitro* mutagenesis of MC4R

Wild type (WT) human (h) MC4R (generously provided by Dr. Ira Gantz) tagged at the N-terminus with c-myc epitope tag was described previously [14]. Mutations were introduced into myc-hMC4R by QuikChange<sup>TM</sup> site-directed mutagenesis kit (Stratagene, La Jolla, CA) [14]. Plasmids used for transfection were prepared using IsoPure Maxi Prep kit (Denville Scientific, Metuchen, NJ). Automated DNA sequencing was used to verify that the full coding region was correct.

### 2.3. Cells and transfections

HEK293T cells, obtained from American Type Culture Collection (Manassas, VA), were maintained at 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium containing 10 mM HEPES, 10% newborn calf serum, 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were plated on gelatin-coated 35 mm 6-well clusters. Cells were transfected using the calcium precipitation method [20]. One  $\mu$ g plasmids in 2 ml media were used per 35 mm dish. Cells were used approximately 48h after transfection for measuring ligand binding and signaling. For confocal microscopy and flow cytometry, stable nonclonal cells were established after transfection of HEK293 cells and selection with G418 as described previously [14, 21].

#### *2.4. Ligand binding to intact cells*

The methods for ligand binding have been described in detail before [14]. Briefly, 48 hours after transfection, cells were washed twice with warm Waymouth/BSA (Waymouth's MB752/1 media, Sigma-Aldrich Cat # W1625, containing 1 mg/ml bovine serum albumin (BSA)) and incubated with 100,000 cpm of <sup>125</sup>I-NDP-MSH with or without different concentrations of unlabeled  $\alpha$ - or  $\beta$ -MSH (from 10<sup>-10</sup> to 10<sup>-5</sup> M) at 37 C for 1 hour. Cells were then placed directly on ice, washed twice with cold Hank's balanced salt solution containing 1 mg/ml BSA. Then 100  $\mu$ l of 0.5 M NaOH was added to each well. Cell lysates were then collected using cotton swabs, and counted in a gamma counter. Apparent maximal binding (Bmax) [22] and IC<sub>50</sub> values were calculated using Prism 4.0 software (San Diego, CA).

#### *2.5. Ligand stimulation of intracellular cAMP production*



HEK293T cells were plated and transfected as described above. Approximately 48 hours after transfection, cells were washed twice with warm Waymouth/BSA. Then 1 ml of fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma-Aldrich, St. Louis, MO) was added to each well. After 15 min incubation at 37 C, either buffer alone or different concentrations of  $\alpha$ - or  $\beta$ -MSH were added and the incubation was continued for another hour. Intracellular cAMP were extracted by the addition of 0.5 N perchloric acid containing 180  $\mu$ g/ml theophylline (to inhibit phosphodiesterase activity), and measured using radioimmunoassay [23]. All determinations were performed in triplicate. Maximal response ( $R_{max}$ ) and  $EC_{50}$  values were calculated using Prism 4.0 software.

#### *2.6. Imaging of cell surface expression of the MC4R by confocal microscopy*

The methods for confocal microscopy have been described in detail before [14, 21]. Briefly, cells stably expressing MC4Rs were plated onto lysine-coated slides (Biocoat cellware from Falcon, BD Systems, Franklin Lakes, NJ) and used for immunostaining 24 h later. All solutions and procedures for immunohistochemistry were at room temperature. Cells were washed three times with filtered phosphate buffered saline for immunohistochemistry (PBS-IH, 137 mM NaCl, 2.7 mM KCl, 1.4 mM  $KH_2PO_4$ , 4.3 mM  $Na_2HPO_4$ , pH 7.4). Cells were then fixed with 4% paraformaldehyde in PBS-IH for 30 min. Then the cells were incubated with blocking solution (5% BSA in PBS-IH) for 1 hour, and incubated 1 hour with monoclonal anti-myc (9E10) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 in PBS-IH containing 1 mg/ml BSA. Cells were then washed three times and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) diluted 1:1000 in PBS-IH containing 1 mg/ml

BSA. Cells were then washed 5 times with PBS-IH and covered with Vectashield Mounting Media (Vector Laboratories) and a glass coverslip. Images were collected with a Bio-Rad laser scanning confocal microscope. Alexa Fluor 488 was excited by a 488-nm argon laser and detected with a 530-560nm filter.

### *2.7. Flow cytometry assay to quantitate expression levels of mutant MC4Rs*

This assay was performed as described in detail before [24, 25] except that the antibodies used and dilutions of antibodies were the same as described above for confocal microscopy. Fluorescence signals were collected with a C6 Accuri Cytometer (Accuri Cytometers, Inc, Ann Arbor, MI). All the steps were performed at room temperature. Fluorescence from cells stably transfected with the empty vector was used to measure background staining. The expression level of the mutants was calculated as a percentage of WT MC4R expression using the formula:  $[\text{mutant fluorescence} - \text{pcDNA3 fluorescence}] / [\text{WT fluorescence} - \text{pcDNA3 fluorescence}] \times 100\%$ .

### *2.8. Statistic analyses*

Ligand binding and signaling data were analyzed using GraphPad Prism 4.0 to calculate  $IC_{50}$ ,  $EC_{50}$ ,  $B_{max}$ , and  $R_{max}$ . The significance of differences in cell surface expression levels and signaling and binding parameters between the WT and mutant hMC4Rs were analyzed using Student's t-test with Prism 4.0.

### 3. Results

#### 3.1. Ligand binding and signaling to $\alpha$ -MSH

The endogenous ligand,  $\alpha$ -MSH, is the most commonly used ligand in MC4R studies. We therefore first studied the binding and signaling properties of the mutant receptors using  $\alpha$ -MSH as the ligand. For binding studies, iodinated NDP-MSH was used because  $\alpha$ -MSH loses the ability to bind to receptors after iodination. HEK293T cells transiently transfected with WT or mutant receptors were incubated with iodinated NDP-MSH in the absence or presence of increasing concentrations of unlabeled  $\alpha$ -MSH. After incubation, cells were washed and iodinated NDP-MSH bound to the cell surface was measured. These experiments showed that unlabeled  $\alpha$ -MSH displaced the radiolabeled NDP-MSH bound to the WT MC4R with an  $IC_{50}$  of 1684.23 nM (Fig. 2A and Table 1). Of the twenty mutants, I69R, D126Y, Del170, I194T, P260Q, and Q307X, had no measurable binding. S94N, D146N, and L300P had decreased maximal binding. Several mutants, including H76R, S94N, D146N, I195S, F280L, and L300P, had lower  $IC_{50}$ s (increased affinities).

When cells transfected with WT or mutant MC4Rs were stimulated with  $\alpha$ -MSH, WT MC4R responded with dose-dependent increases in intracellular cAMP levels, with an  $EC_{50}$  of 2.59 nM (Fig. 2B and Table 1). Six mutants, I69R, D126Y, Del170, I194T, P260Q, and Q307X, had no response to  $\alpha$ -MSH stimulation, consistent with their lack of detectable binding. M79I, despite normal ligand binding, had decreased maximal response and increased  $EC_{50}$  suggesting that this mutant was partially defective in signaling. The other mutants could signal at least as well as WT

MC4R in terms of maximal signaling. R305S even had significantly increased maximal response. Several mutants, including D146N, I289L, Y332C, and Y332H, had decreased  $EC_{50}$ s (Fig. 2B and Table 1).

### 3.2. Ligand binding and signaling to $\beta$ -MSH

Although  $\alpha$ -MSH is usually used in MC4R studies, human genetic studies suggest that  $\beta$ -MSH might also be an important endogenous ligand, especially in humans [26, 27]. We therefore also studied the binding and signaling properties of the mutant receptors using  $\beta$ -MSH as the ligand.

Similar to the experiments described above for  $\alpha$ -MSH, increasing concentrations of unlabeled  $\beta$ -MSH were used to displace iodinated NDP-MSH bound to cells expressing WT or mutant MC4Rs. These experiments showed that unlabeled  $\beta$ -MSH displaced the radiolabeled NDP-MSH with an  $IC_{50}$  of 1506.32 nM (Fig. 3A and Table 2). Similar to the data when  $\alpha$ -MSH was used as the ligand, four mutants, including H76R, S94N, D146N, and L300P, had lower  $IC_{50}$ s (increased affinities).

Stimulation with  $\beta$ -MSH in WT MC4R resulted in dose-dependent increases in intracellular cAMP levels, with an  $EC_{50}$  of 3.24 nM (Fig. 3B and Table 2). Data obtained from the mutants were similar to the data obtained when  $\alpha$ -MSH was used as the agonist, with no signaling for I69R, D126Y, Del170, I194T, P260Q, and Q307X. M79I had decreased maximal response and increased  $EC_{50}$ . The other mutants could signal at least as well as WT MC4R in terms of

maximal signaling. Several mutants, including D146N, I186V, and I289L, had decreased  $EC_{50}$ s (Fig. 3B and Table 2).

Since one previous study suggested that basal signaling might be important for maintaining energy homeostasis in humans [28], we also measured the basal signaling of the mutant MC4Rs. As shown in Fig. 4, three mutants, H76R, D146N, and F280L, had significantly increased basal signaling. Of the remaining 17 mutants, 11 had decreased basal signaling, mostly due to decreased or absent cell surface expression. Three mutants, M79I, F201L, and R305S, had relatively normal cell surface expression but decreased basal signaling. I186V, I195S, G231V, I289L, Y332C, and Y332H, had normal levels of basal signaling (Fig. 4).

### 3.3. Expression levels of the mutant receptors

Previous studies suggested that defective trafficking of mutant receptors to the cell surface is the predominant defect in naturally occurring *MC4R* mutations (reviewed in [15]). To image cell surface expression, HEK293 cells stably expressing WT or mutant receptors were immunostained with anti-myc monoclonal antibody 9E10 and imaged with a confocal microscope. As shown in Fig. 5A, confocal microscopy showed that WT MC4R was expressed well on the cell surface. Some mutants, such as M79I, D126Y, I186V, F201L, G231V, I289L, R305S, Y332C, and Y332H, were expressed well on the cell surface. The other mutants had either decreased or absent cell surface expression. To quantitate the cell surface expression, flow cytometry was used. These results showed that 11 mutants had either absent or significantly

decreased cell surface expression compared with WT MC4R (Fig. 5B). The expressions levels of the other mutants were not significantly different from that of the WT receptor (Fig. 5B).

To measure the total expression of the MC4Rs, cells were permeabilized and total expression measured by flow cytometry. As shown in Fig. 5C, four mutants, including M79I, S94N, D146N, and Del170, had significantly decreased total expression. The other 16 mutants had normal expression levels as the WT MC4R.

#### 4. Discussion

In the present study, we performed detailed functional studies on 18 naturally occurring *MC4R* mutations reported recently that were either incompletely characterized or not characterized. Analogous mutations corresponding to two mutations identified from obese mouse lines were also characterized. We studied the pharmacology of these mutant receptors using both of the endogenous ligands,  $\alpha$ - and  $\beta$ -MSH. Table 3 summarized the data obtained. We showed that 4 mutants, M79I, S94N, D146N, and Del170, had significantly decreased total expression levels as measured by flow cytometry (Fig. 5C). These 4 mutants are Class I mutants. Six mutants, including I69R, D126Y, Del170, I194T, P260Q, and Q307X, had little or no binding to radiolabeled ligand (Fig. 2A and 3A). Confocal microscopy and flow cytometry showed that all mutants except D126Y had decreased or absent cell surface expression (Fig. 5A and 5B). In addition to these five mutants, six additional mutants, including H76R, S94N, D146N, I195S, F280L, and L300P, had decreased cell surface expression (Fig. 5A and 5B). These 11 mutants are Class II mutants (Table 3). We and others recently showed that small molecule MC4R

antagonists as well as some chemical chaperones could rescue some of the intracellularly retained mutants to the cell surface and restore function [2, 25, 29, 30]. Whether these agents can rescue these mutants remains to be investigated.

Aspartic acid 126 sits at the top of the third transmembrane domain. We showed that D126Y was expressed normally at the cell surface but no ligand binding or signaling could be measured. These results are consistent with previous mutagenesis experiments demonstrating D126 as being important for binding to peptide [31] and nonpeptide [32] ligands, hypothesized to interact with the arginine side chain of  $\alpha$ -MSH [33]. The corresponding Asp is also important for ligand binding in the MC3R [24, 34]. Of all the naturally occurring *MC4R* mutations that were carefully characterized, only a few have been shown to be defective in ligand binding *per se* [21, 35, 36], although some of the intracellularly retained mutants might also be defective in ligand binding. D126Y joins the list as a Class III mutant.

We previously reported H76R and D146N had increased basal activities [37]. Three *MC4R* antagonists, including the endogenous AgRP and two small molecule synthetic antagonists, ML00253764 and Ipsen 5i, were shown to be inverse agonists for these two mutant receptors as well as the WT *MC4R* [37]. AgRP is a full inverse agonist whereas ML00253764 and Ipsen 5i are partial inverse agonists [37]. We found herein F280L also had increased basal activities. In addition to the increased basal activities, the mutants had similar maximal signaling in response to  $\alpha$ - and  $\beta$ -MSH stimulation (Fig. 2B and 3B) with decreased  $EC_{50}$ s (Tables 1 and 2). These properties are not consistent with their potential roles in obesity pathogenesis [38]. However, all three mutants had decreased cell surface expression (Fig. 5), which is likely the cause for

decreased signaling *in vivo*, as previously suggested for L250Q [39], another constitutively active mutant. One caveat for the *in vitro* expression system is the presence of spare receptors, unlikely to occur *in vivo* [15]. The reason for the decreased cell surface expression, either due to defective forward trafficking or accelerated constitutive internalization/defective recycling of receptors to the cell surface resulting in down-regulation, remains to be investigated. Defective trafficking is common in naturally occurring MC4R mutations [14, 35, 40, 41] (reviewed in [42]). The MC4R is also known to undergo constitutive endocytosis [43]. Other potential reasons for constitutively active mutants in causing obesity are constitutive desensitization and induction of phosphodiesterase *in vivo* (therefore increased cAMP degradation) as previously observed with other constitutively active GPCR mutants [44, 45]. Phosphodiesterase inhibitors are included in the *in vitro* experiments described herein. Further studies are needed to clarify the mechanism for the decreased cell surface expression of these mutants. These studies will be important basis for potential personalized medicine to treat patients harboring these mutations.

The WT MC4R has some basal signaling. It was suggested that decreased basal signaling could cause obesity [28]. We showed that of the 17 mutants (excluding the three constitutively active mutants), 11 had decreased basal signaling, mostly due to decreased or absent cell surface expression. Only three mutants, M79I, F201L, and R305S, had relatively normal cell surface expression but decreased basal signaling. M79I also had decreased maximal responses to  $\alpha$ - and  $\beta$ -MSH stimulation but F201L and R305S had normal maximal response. If indeed basal signaling was important for energy homeostasis, F201L and R305S might cause obesity due to defective basal signaling. Further studies are needed to confirm this hypothesis. We suggest that these three mutants are Class IV mutants.



I194T and L300P were identified in obese mouse lines generated by *N*-ethyl-*N*-nitrosourea mutagenesis [19]. Functional studies with mouse MC4R showed that I194T mouse (m) MC4R has decreased cell surface expression, ligand binding, and increased EC<sub>50</sub> in response to  $\alpha$ -MSH and NDP-MSH stimulation, whereas L300P mMC4R has normal cell surface expression but no ligand binding and signaling [19]. We showed here that hMC4R I194T had decreased cell surface expression, no measurable ligand binding or signaling to either  $\alpha$ -MSH or  $\beta$ -MSH stimulation. Basal signaling was also significantly decreased. For hMC4R L300P, the cell surface expression was decreased, with corresponding decrease in ligand binding. However, signaling to both  $\alpha$ -MSH and  $\beta$ -MSH stimulation was similar as the WT hMC4R, although basal signaling was significantly decreased as compared to the WT hMC4R. These results suggest that there are important differences in the functions of the two conserved codons in human and mouse MC4R. The molecular basis for these differences remains to be identified. In human MC4R, both mutants are Class II mutants. It is interesting to note that in the MC3R, mutation in an isoleucine residue next to the analogous position of L300 was also found to cause intracellular retention [46, 47].

Five mutants, including I186V, G231V, I289L, Y332C, and Y332H, had normal cell surface expression, ligand binding and signaling. Therefore they are Class V mutants, mutants with unknown defects. Whether these mutations are indeed the cause of obesity in individuals harboring these mutations, and if yes, what are the molecular mechanism(s), are not clear at present.

In summary, of the 20 mutations we studied herein, 11 (55%) had significantly decreased cell surface expression, consistent with previous studies suggesting that intracellular retention is the most common defect in naturally occurring *MC4R* mutations. Many of these mutants are likely defective in forward trafficking. Decreased cell surface expression of the three constitutively active mutants, H76R, D146N, and F280L, might be due to defective forward trafficking and/or accelerated internalization/inefficient recycling of internalized receptors. We also identified mutants that are defective in ligand binding per se (D126Y) or signaling (M79I, F201L, and R305S). Five mutants had normal properties compared to the WT *MC4R*. Further studies are needed to identify potential therapeutic approaches for correcting the defects of the individual mutations.

**Acknowledgements**

We thank Dr. Ira Gantz for generously providing the wild type MC4R construct, Dr. Robert C. Speth for providing the iodinated NDP-MSH at low cost, and Dr. Shuxiu Wang for generating some of the mutant constructs. This study was supported by grants from the National Institutes of Health R15DK077213 and Animal Health and Diseases Research Program as well as Interdisciplinary Grant from Auburn University College of Veterinary Medicine. Z-Q Wang was partially supported by the Education Commission of Jiangsu Province, P. R. China.

**Disclosure**

None.

**Figure legends:**

**Figure 1.** Schematic model of the hMC4R with the mutations studied in this study highlighted with gray background. All are naturally occurring mutations identified in humans except that mutations corresponding to I194T and L300P were identified in obese mouse lines. The c-myc epitope at the N terminus are highlighted with white letters on dark background.

**Figure 2.** Ligand binding and signaling properties of the WT and mutant hMC4Rs with  $\alpha$ -MSH as the ligand. HEK293T cells were transiently transfected with the indicated hMC4R constructs and binding and signaling properties of the MC4Rs were measured as described in Materials and methods. In A, different concentrations of unlabeled  $\alpha$ -MSH were used to displace the binding of  $^{125}$ I-NDP-MSH to hMC4Rs on intact cells. Results shown are expressed as % of WT binding  $\pm$  range from duplicate determinations within one experiment. In B, HEK293T cells transiently transfected with the indicated hMC4R constructs were stimulated with different concentrations of  $\alpha$ -MSH. Intracellular cAMP levels were measured using RIA. Results are expressed as the mean  $\pm$  SEM of triplicate determinations within one experiment. All experiments were performed three times.

**Figure 3.** Ligand binding and signaling properties of the WT and mutant hMC4Rs with  $\beta$ -MSH as the ligand. See the legend to Fig. 2 for details. The only difference is that  $\beta$ -MSH was used as the ligand instead of  $\alpha$ -MSH.

**Figure 4.** Constitutive activities of the WT and mutant hMC4Rs. Intracellular cAMP levels in cells transiently transfected with WT or mutant hMC4Rs were measured in the absence of any ligand. The results are expressed as fold over WT basal activity. Shown are mean  $\pm$  SEM of 6 experiments for the mutant hMC4Rs. The basal cAMP levels in cells expressing WT MC4R were  $36.96 \pm 4.58$  pmol/ $10^6$  cells (mean  $\pm$  SEM of 21 experiments). The statistical significance are indicated as follows: a: significantly different from WT hMC4R,  $p < 0.05$ ; b: significantly different from WT hMC4R,  $p < 0.01$ ; c: significantly different from WT hMC4R,  $p < 0.001$ .

**Figure 5.** Cell surface expression of WT and mutant hMC4Rs. A. Confocal imaging of WT and mutant hMC4Rs. The WT or mutant myc-MC4Rs stably expressed in HEK293 cells were stained with fluorescein-conjugated anti-myc monoclonal antibody and imaged by confocal microscopy. This experiment was done twice with similar results. B. Cell surface expression of the WT and mutant hMC4Rs were measured by flow cytometry. The results are expressed as percentage of cell surface expression levels of the WT hMC4R after correction of the nonspecific staining in cells stably transfected with the empty vector as described in Materials and methods. Data were mean  $\pm$  SEM of 4-5 experiments. The statistical significance are indicated as follows: a: significantly different from WT hMC4R,  $p < 0.05$ ; b: significantly different from WT hMC4R,  $p < 0.01$ ; c: significantly different from WT hMC4R,  $p < 0.001$ . C. Total expression of the WT and mutant hMC4Rs were measured by flow cytometry. The results are expressed as percentage of total expression levels of the WT hMC4R after correction of the nonspecific staining in cells stably transfected with the empty vector as described in Materials and methods. Data were mean  $\pm$  SEM of 4 experiments. The statistical significance are indicated as follows: a: significantly different from WT hMC4R,  $p < 0.05$ ; c: significantly different from WT hMC4R,  $p < 0.001$ .

**Table 1  $\alpha$ -MSH-stimulated signaling and ligand binding of WT and mutant hMC4Rs**

hMC4R	n	$\alpha$ -MSH Binding		$\alpha$ -MSH-stimulated cAMP	
		IC <sub>50</sub> (nM)	B <sub>max</sub> (% wt)	EC <sub>50</sub> (nM)	R <sub>max</sub> (% wt)
WT	12	1684.23±412.59	100	2.59±0.30	100
I69R	3	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
H76R	3	41.31±18.97 <sup>a</sup>	151.05±4.21 <sup>b</sup>	0.92±0.38	158.55±39.78
M79I	3	328.30±75.53	97.20±15.39	153.10±14.83 <sup>c</sup>	59.84±2.99 <sup>b</sup>
S94N	3	53.70±11.70 <sup>a</sup>	53.35±8.96 <sup>a</sup>	5.26±0.66	218.48±44.69
D126Y	3	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
D146N	3	4.41±1.06 <sup>a</sup>	52.76±12.65	0.08±0.01 <sup>b</sup>	133.47±31.68
Del170	3	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
I186V	3	1084.70±75.18	93.25±17.13	0.60±0.21	107.15±28.47
I194T	3	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
I195S	3	329.08±105.12 <sup>a</sup>	113.43±14.23	3.11±0.82	282.55±66.91
F201L	3	1224.10±415.47	78.64±7.53	8.41±0.09 <sup>b</sup>	108.88±2.11
G231V	3	745.70±35.64	147.07±15.51	1.74±0.44	149.08±14.00
P260Q	3	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
F280L	3	150.10±15.10 <sup>a</sup>	82.50±18.20	0.64±0.15	174.40±33.59
I289L	3	1866.33±54.87	118.41±12.34	0.55±0.17 <sup>a</sup>	91.09±12.82
L300P	3	53.60±0.73 <sup>a</sup>	63.75±11.93	3.30±1.34	204.16±37.75
R305S	3	2263.67±503.38	133.66±20.88	8.75±1.07 <sup>b</sup>	180.56±5.59 <sup>b</sup>
Q307X	3	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
Y332C	3	957.03±398.64	163.97±14.75	0.34±0.07 <sup>a</sup>	106.07±3.14
Y332H	3	1674.37±552.71	169.06±24.09	0.76±0.21 <sup>a</sup>	129.92±26.91

The data are expressed as the mean  $\pm$  SEM of three independent experiments for the mutant hMC4Rs. The maximal responses ( $R_{max}$ ) were 2572.42±281.39 pmol cAMP/10<sup>6</sup> cells for WT hMC4R.

<sup>a</sup> Significantly different from corresponding WT receptor,  $p < 0.05$ .

<sup>b</sup> Significantly different from corresponding WT receptor,  $p < 0.01$ .

<sup>c</sup> Significantly different from corresponding WT receptor,  $p < 0.001$ .

<sup>d</sup> ND, could not be detected.

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Table 2  $\beta$ -MSH-stimulated signaling and ligand binding of WT and mutant hMC4Rs

hMC4R	n	$\beta$ -MSH Binding		$\beta$ -MSH-stimulated cAMP	
		IC <sub>50</sub> (nM)	B <sub>max</sub> (% wt)	EC <sub>50</sub> (nM)	R <sub>max</sub> (% wt)
WT	9	1506.32±272.82	100	3.24±0.59	100
I69R	3	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
H76R	3	111.34±13.30 <sup>a</sup>	81.74±5.94	1.18±0.29	78.40±7.29
M79I	3	632.67±221.03	98.24±10.01	283.97±149.04 <sup>b</sup>	51.64±12.36
S94N	3	239.64±100.82 <sup>a</sup>	40.97±12.24 <sup>a</sup>	7.33±1.37 <sup>a</sup>	166.81±38.56
D126Y	3	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
D146N	3	66.74±31.45 <sup>a</sup>	23.85±10.97 <sup>a</sup>	0.47±0.18 <sup>a</sup>	96.11±23.80
Del170	3	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
I186V	3	1106.90±308.77	150.52±55.35	1.06±0.22 <sup>a</sup>	65.81±10.68
I194T	3	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
I195S	3	593.40±159.61	71.15±14.57	4.28±1.16	94.06±22.30
F201L	3	866.97±185.25	115.98±25.83	5.30±0.63 <sup>a</sup>	99.95±33.34
G231V	3	1450.20±392.34	123.15±14.33	1.77±0.23 <sup>a</sup>	104.94±24.02
P260Q	3	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
F280L	3	536.50±188.87	57.41±23.96	1.68±0.34	129.79±24.22
I289L	3	2294.67±604.62	127.39±31.88	1.26±0.11 <sup>b</sup>	76.75±24.97
L300P	3	342.63±126.95 <sup>a</sup>	21.96±3.34 <sup>a</sup>	5.83±1.11 <sup>a</sup>	124.05±31.36
R305S	3	1327.40±421.20	128.06±27.67	6.92±1.06 <sup>a</sup>	108.98±20.76
Q307X	3	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>	ND <sup>d</sup>
Y332C	3	832.13±140.24	129.62±18.23	1.55±0.39	93.37±19.00
Y332H	3	975.93±75.98	149.98±42.91	2.54±1.48	61.29±18.59

The data are expressed as the mean  $\pm$  SEM of three independent experiments for the mutant hMC4Rs. The maximal responses ( $R_{max}$ ) were 3535.56±463.44 pmol cAMP/10<sup>6</sup> cells for WT hMC4R.



<sup>a</sup> Significantly different from corresponding WT receptor,  $p < 0.05$ .

<sup>b</sup> Significantly different from corresponding WT receptor,  $p < 0.01$ .

<sup>c</sup> Significantly different from corresponding WT receptor,  $p < 0.001$ .

<sup>d</sup> ND, could not be detected

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**Table 3 Summary of the functional properties of the twenty mutants studied herein**

MC4R	Surface expression <sup>a</sup>	Binding		Signaling		Class <sup>b</sup>	
		$\alpha$ -MSH	$\beta$ -MSH	Basal	Stimulated		
				$\alpha$ -MSH	$\beta$ -MSH		
I69R	↓	↓	↓	↓	↓	↓	II
H76R	↓	+	+	↑	+	+	II
M79I	+	+	+	↓	↓	↓	I
S94N	↓	↓	↓	↓	+	+	I
D126Y	+	↓	↓	↓	↓	↓	III
D146N	↓	↓	↓	↑	+	+	I
Del170	↓	↓	↓	↓	↓	↓	I
I186V	+	+	+	+	+	+	V
I194T	↓	↓	↓	↓	↓	↓	II
I195S	↓	+	+	+	+	+	II
F201L	+	+	+	↓	+	+	IV <sub>a</sub>
G231V	+	+	+	+	+	+	V
P260Q	↓	↓	↓	↓	↓	↓	II
F280L	↓	+	+	↑	+	+	II
I289L	↑	+	+	+	↑	↑	V
L300P	↓	↓	↓	↓	+	+	II
R305S	+	+	+	↓	+	+	IV <sub>a</sub>
Q307X	↓	↓	↓	↓	↓	↓	II
Y332C	+	+	+	+	+	+	V
Y332H	+	+	+	+	+	+	V

+: Denotes the particular function is normal.

<sup>a</sup>: Cell surface expression was quantitatively assessed by flow cytometry.

<sup>b</sup>: The classification of the MC4R mutants are based on the scheme we proposed earlier. IV<sub>a</sub> mutants are defective only in basal signaling but not stimulated signaling.

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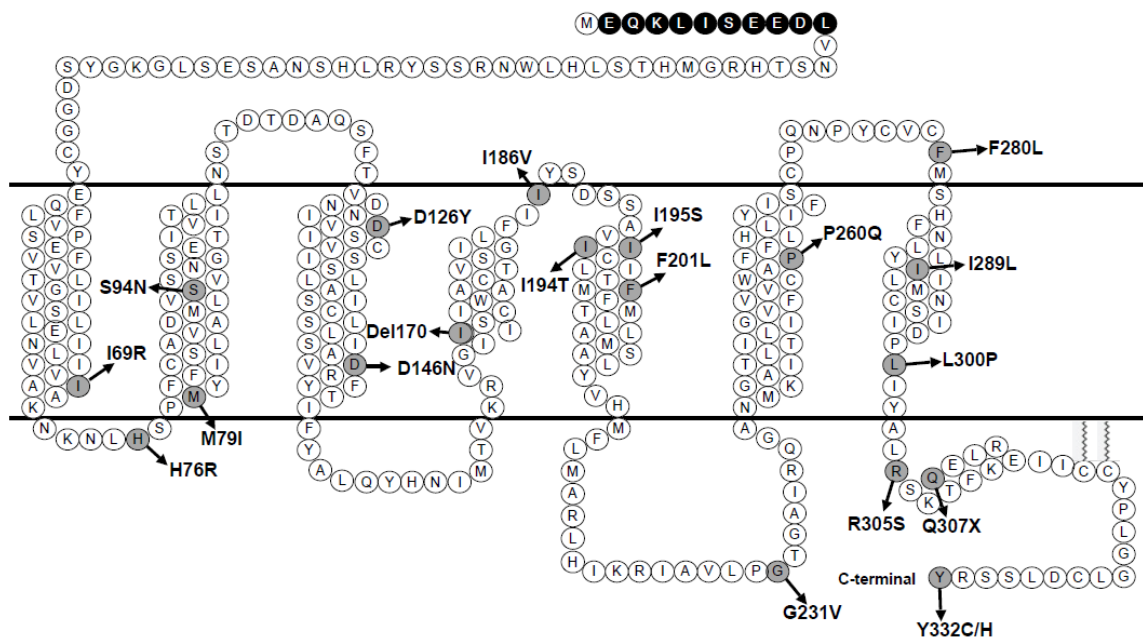
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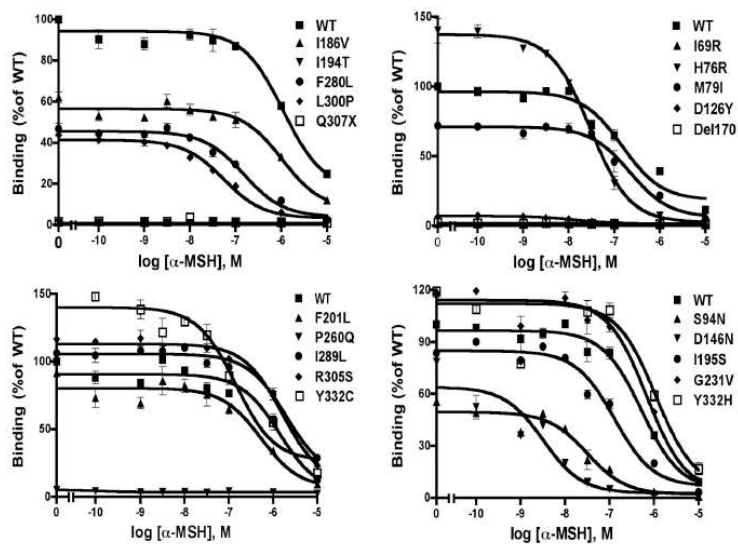


Fig. 1 Wang &amp; Tao



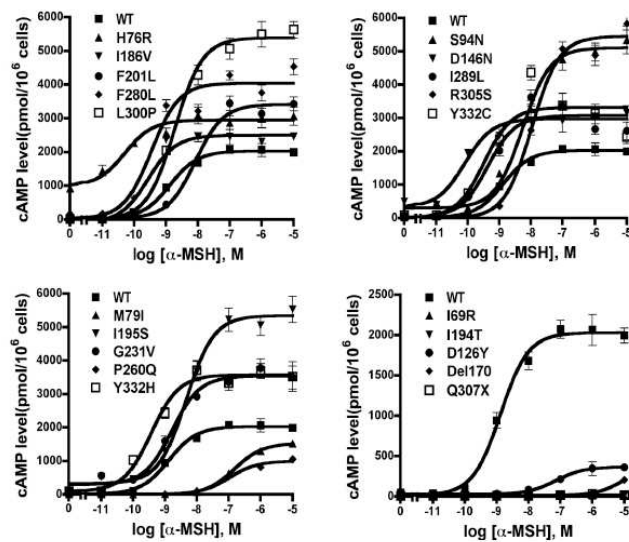
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Fig. 2A Wang &amp; Tao



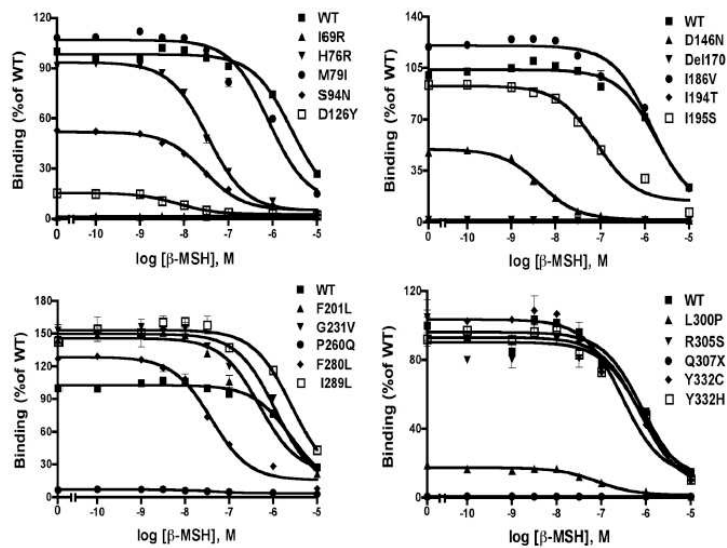
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Fig. 2B Wang &amp; Tao



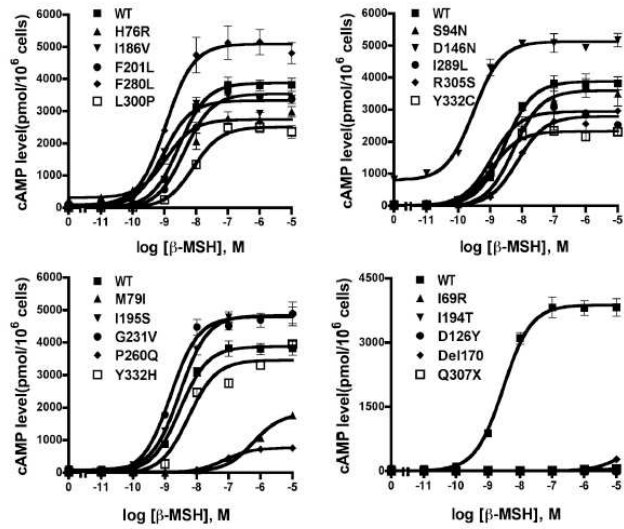
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Fig. 3A Wang &amp; Tao



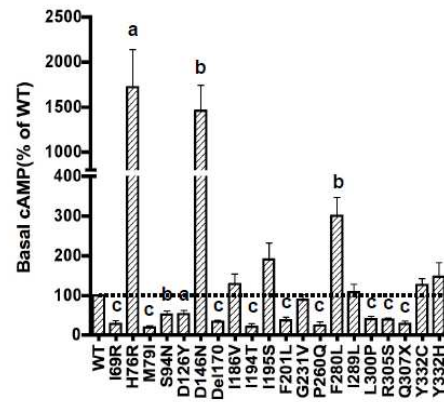
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Fig. 3B Wang &amp; Tao



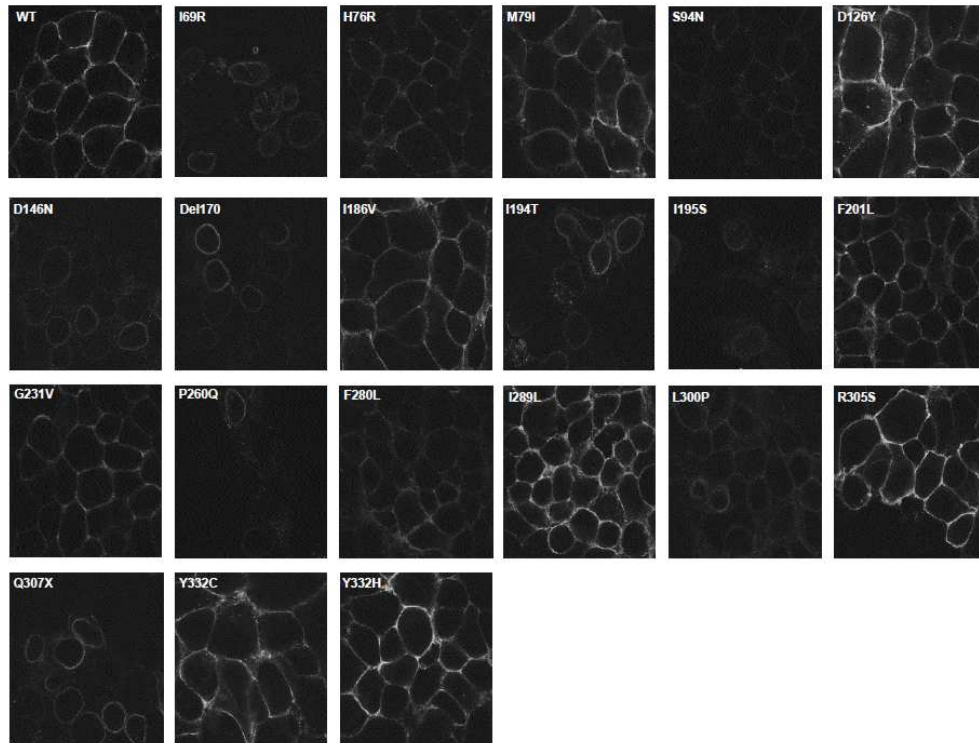
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Fig. 4 Wang &amp; Tao



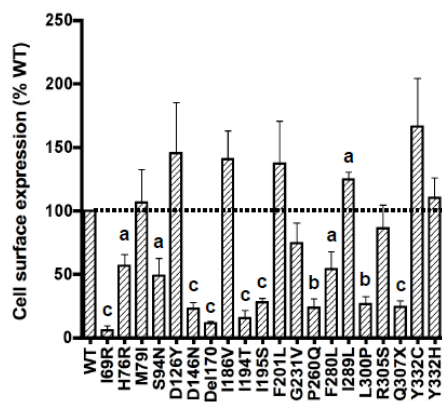
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Fig. 5A Wang &amp; Tao



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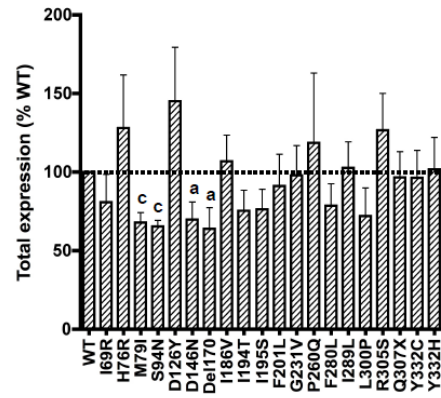
Fig. 5B Wang &amp; Tao



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Fig. 5C Wang &amp; Tao



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## Highlights

We performed detailed functional studies on 20 naturally occurring MC4R mutations.

More than half of the mutants had decreased cell surface expression.

We identified a mutant defective in ligand binding per se.

Constitutively active mutations had decreased cell surface expression.

Some mutants have normal functions.

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