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# Defect in MAPK Signaling As a Cause for Monogenic Obesity Caused By Inactivating Mutations in the Melanocortin-4 Receptor Gene

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### Abstract

The melanocortin-4 receptor (MC4R) is a Family A G protein-coupled receptor that plays an essential role in regulating energy homeostasis, including both energy intake and expenditure. Mutations leading to a reduced MC4R function confer a major gene effect for obesity. More than 170 distinct mutations have been identified in humans. In addition to the conventional Gs-stimulated cAMP pathway, the MC4R also activates MAPKs, especially ERK1/2. We also showed there is biased signaling in the two signaling pathways, with inverse agonists in the Gs-cAMP pathway acting as agonists for the ERK1/2 pathway. In the current study, we sought to determine whether defects in basal or agonist-induced ERK1/2 activation in MC4R mutants might potentially contribute to obesity pathogenesis in patients carrying these mutations. The constitutive and ligand-stimulated ERK1/2 activation were measured in wild type and 73 naturally occurring MC4R mutations. We showed that nineteen mutants had significantly decreased basal pERK1/2 level, and five Class V variants (where no functional defects have been identified previously), C40R, V50M, T112M, A154D and S295P, had impaired ligand-stimulated ERK1/2 activation. Our studies demonstrated for the first time that decreased basal or ligand-stimulated ERK1/2 signaling might contribute to obesity pathogenesis caused by mutations in the MC4R gene. We also observed biased signaling in 25 naturally occurring mutations in the Gs-cAMP and ERK1/2 pathways.

Key words: Melanocortin-4 receptor; naturally occurring mutation; extracellular signal-regulated kinases 1 and 2 signaling; biased signaling.

## Introduction

The melanocortin-4 receptor (MC4R) is a member of Family A G protein-coupled receptors (GPCRs) that has been shown to be involved in regulating energy homeostasis, including both energy intake and expenditure [1-3]. Human genetic studies demonstrated that functionally relevant mutations in the *MC4R* confer the commonest major gene form of obesity, characterized by its early-onset and severity [4]. About 170 *MC4R* mutations, including nonsense, missense, frameshift, and inframe deletions, have been identified, primarily from study groups of obese patients of different ethnic origins [5, 6]. We grouped *MC4R* mutations into five Classes [7]. Class I are null mutations that are defective in receptor biosynthesis. Class II mutants are synthesized but defective in trafficking onto the cell surface. Class III mutants are

synthesized and transported onto the cell surface, but are defective in ligand binding. Class IV mutants have normal cell surface expression and ligand binding but are defective in Gs-mediated cAMP signaling. Class V variants have no known defects with normal cell surface expression, ligand binding and cAMP signaling [7]. The obesity observed *in vivo* in the patients could not be well explained by the *in vitro* cellular phenotype with Class V variants. Therefore, the analysis of the *in vitro* signaling defects of these mutations is necessary to understand its potential roles in obesity pathogenesis.

When the MC4R was cloned, it was shown to be coupled to the stimulatory G protein, increasing adenylyl cyclase activity and intracellular cAMP levels [8, 9] (reviewed in [10]). Indeed, almost all of the earlier studies on the MC4R used direct or indirect measures of cAMP levels as the indicator for receptor activation. However, like other GPCRs, the MC4R has been found to activate other G proteins, including the inhibitory G protein (Gi) and signaling pathways, including MAPK, such as ERK1/2, p38 MAPK, and c-Jun NH2-terminal kinase [3, 11, 12]. In vitro experiments in different cell lines expressing MC4R heterologously or GT1-1 cells that express mouse MC4R endogenously showed that agonist stimulation of MC4R activates ERK1/2, which can be mediated by cAMP-protein kinase phosphatidylinositol Α,

3-kinase, calcium, and protein kinase C, depending on the cell lines used [13-16]. *In vivo*, activation of ERK1/2 by MC4R is also observed in the hypothalamus, and this signaling is involved in mediating melanotan II-induced inhibition of food intake [14, 17]. Therefore, defective ERK1/2 signaling might be involved in obesity pathogenesis caused by mutations in the *MC4R* gene.

Previous studies discovered biased activation of ERK1/2 in MC4R with several artificially generated and one naturally occurring MC4R mutations having divergent basal or agonist-stimulated cAMP and ERK1/2 signaling [12, 18-20]. We also showed that several inverse agonists at the Gs-cAMP pathway are indeed agonists at the ERK1/2 pathway, suggesting that these ligands are biased [16]. However, little is known about the potential dysfunction in ERK1/2 signaling in naturally occurring MC4R mutations, and their contributions to obesity pathogenesis in patients carrying these mutations. In the present study, we investigated the constitutive and ligand-stimulated activation of ERK1/2 in wild type (WT) and 73 naturally occurring MC4R mutations from all five Classes (Fig. 1). By comparing the signaling properties of the mutant receptors in the Gs-cAMP pathway previously reported and the ERK1/2 data obtained in the current study, we were interested in whether there is biased signaling in the naturally occurring MC4R mutations.



Figure 1. Schematic model of the human MC4R. The naturally occurring mutations characterized in this study are highlighted with gray background. The variants that had impaired ligand-stimulated ERK1/2 activation in Class V are highlighted with red background. The mutations that had biased signaling in the Gs-cAMP and ERK1/2 pathways are highlighted with blue background.

Biased signaling, also called functional selectivity, agonist-directed stimulus trafficking, or ligand-induced differential signaling, is a very actively studied area in GPCR field, representing a frontier in GPCR pharmacology and drug discovery [21-26]. Biased ligands with enhanced therapeutic potential and diminished side effects targeting several GPCRs are in various stages of clinical trials. The atomic basis of biased signaling is also beginning to be elucidated with crystal structure analysis [27-30]. Not only ligands can be biased, mutant receptors can also be biased [24, 31]. In addition to lab-generated mutations that show biased signaling (see [32] for an example), naturally occurring mutations in several GPCRs including glucagon-like peptide-1 receptor, calcium-sensing receptor, melanocortin-1 receptor, and MC4R, have also been shown to exhibit biased signaling [12, 33-36]. With the MC4R, only one mutation, D90N, was shown previously to have biased signaling [12]. Therefore our data on 73 mutations would expand this observation significantly.

## **Methods and Materials**

### **Reagents and supplies**

[Nle<sup>4</sup>,D-Phe<sup>7</sup>]- $\alpha$ -MSH (NDP- $\alpha$ -MSH) was purchased from Peptides International (Louisville, KY). Cell culture plates and flasks were purchased from Corning (Corning, NY). Cell culture media, newborn calf serum, antibiotics and reagents were obtained from Invitrogen (Carlsbad, CA).

### **Plasmids**

The WT human MC4R in pcDNA3.1 was previously described [37]. Mutant MC4Rs used in this study were generated using QuikChange<sup>TM</sup> site-directed mutagenesis kit (Stratagene, La Jolla, CA) and have been reported in our previous studies [7, 37-43]. Plasmids prepared were sequenced by the DNA Sequencing Facility of University of Chicago Cancer Research Center (Chicago, IL, USA) before used for transfection experiments.

#### Cell culture and DNA transfection

HEK293T cells were obtained from the American Type Culture Collection (Manassas, VA), and maintained at 5% CO<sub>2</sub> in DMEM containing 10 mM HEPES, 10% newborn calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B and 50 µg/ml gentamicin. Cells were plated on gelatin-coated 100 mm dishes and transfected at 50–70% confluency using calcium phosphate transfection method. Then cells were washed twice and incubated with Waymouth/BSA (Waymouth's MB752/1 media (Sigma-Aldrich, St. Louis, MO) containing 1 mg/ml bovine serum albumin (BSA)) 24 h after transfection and starved for 18 h at 37 °C before ligand stimulation.

### ERKI/2 phosphorylation assay

The phosphorylated ERK1/2 (pERK1/2) activity was measured as described previously [16, 19, 20]. Briefly, 48 h after transfection, starved cells (see above) were treated for 5 min with either buffer alone or 1  $\mu$ M NDP- $\alpha$ -MSH. The time point in the experiments was chosen based on previous report of MC4R-mediated ERK1/2 signaling [16]. Cells were solubilized in lysis buffer and lysates were separated on 10% SDS-PAGE gel. Proteins were then transferred onto PVDF membrane. Phospho-ERK1/2 and  $\beta$ -tubulin were detected by immunoblotting with rabbit anti-pERK1/2 antibody (1:1000~1:2000, Cell Signaling Technology, Beverly, MA) and mouse anti-B-tubulin antibody (1:5000~1:10,000, Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA), respectively. Blots were probed with horseradish peroxidase-conjugated secondary donkey anti-rabbit (1:2000)and horseradish peroxidase-conjugated donkey anti-mouse (1:5000~1:10,000, both from Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 2 h. The membranes were then visualized using enhanced chemiluminescence reagent (Pierce, Rockford, IL) and quantified using ImageJ 1.44 software (National Institute of Health, Bethesda, MD) after densitometric scanning of the films. ERK1/2 phosphorylation was normalized according to the loading of proteins by expressing the data as a ratio of pERK1/2 over β-tubulin.

### **Statistics**

The significance of differences in pERK1/2 activities between WT and mutant MC4Rs were analyzed using paired two-tailed Student's t-test with GraphPad Prism 4.0 software (La Jolla, CA).

## Results

# Ligand-stimulated activation of ERK1/2 in Class V MC4R variants

HEK293T cells, which do not express MC4R endogenously [37], have been widely used as a mammalian expression system in MC4R studies. We used HEK293T cells transiently transfected with the WT or 73 naturally occurring *MC4R* mutations to study the effect of NDP-α-MSH on MAPK pathway. No significant change of pERK1/2 level was observed upon NDP-α-MSH treatment in HEK293T cells transiently transfected with the empty vector pcDNA3.1 (data not shown). The pERK1/2 levels of the 30 Class V variant MC4Rs, where no functional defects were identified previously, including R7C, T11A, S30F, Y35C, D37V, C40R, P48S, V50M, F51L, T112M, A154D, H158R, I170V, M200V, F201L, F202L, M208V, M218T, G231S, G231V, N240S, G252S, N274S, I289L, S295P, R305S, I317V, L325F, Y332C and Y332H, were measured after 5 min stimulation with NDP-α-MSH. As shown in Figs. 2 and 3, we found that C40R and A154D did not respond to NDP-α-MSH stimulation in ERK1/2 activation. Although the pERK1/2 levels of V50M, T112M, and S295P MC4Rs were increased significantly upon NDP-α-MSH stimulation (P < 0.05), the stimulation was significantly lower than that of the WT MC4R (P < 0.05). The other 25 Class V variants showed similar agonist-stimulated ERK1/2 activation as the WT MC4R (P < 0.05) (Figs. 2 and 3).

# Ligand-stimulated activation of ERK1/2 in Class IV MC4R mutants

The pERK1/2 levels of the four mutant MC4Rs in Class IV (mutants that are expressed on the cell surface and can bind the ligand but are defective in Gs-cAMP signaling), including D90N, S136F, A175T and C326R, were examined after 5 min stimulation with NDP- $\alpha$ -MSH in HEK293T cells transiently transfected with these MC4R mutants. As shown in Fig. 4, A175T and C326R MC4Rs had significant ligand-stimulated ERK1/2 activation, similar to that of the WT MC4R (P < 0.05). D90N and S136F did not

respond to NDP- $\alpha$ -MSH stimulation with ERK1/2 activation.



Figure 2. A representative blot of ligand-stimulated ERK I/2 activation in HEK293T cells transiently transfected with Class V variants.







Figure 4. The ligand-stimulated pERK1/2 levels in HEK293T cells transiently transfected with Class IV mutants. Results are expressed as percentage of the value obtained in non-stimulated cells and represent the mean  $\pm$  SEM of five independent experiments. \* indicates significant differences from basal pERK1/2 level (P < 0.05).



Figure 5. The ligand-stimulated pERK1/2 levels in HEK293T cells transiently transfected with Class III mutants. Results are expressed as percentage of the value obtained in non-stimulated cells and represent the mean  $\pm$  SEM of five independent experiments. \* indicates significant differences from basal pERK1/2 level (P < 0.05).

# Ligand-stimulated activation of ERK1/2 in Class III MC4R mutants

The pERK1/2 levels of six Class III mutant MC4Rs (mutants that are expressed on the cell surface but have defects in ligand binding), including G55V,  $\Delta$ 88-92, I102T, L106P, D126Y, and A219V, were measured after 5 min stimulation with NDP- $\alpha$ -MSH in HEK293T cells transiently transfected with these MC4R mutants. As shown in Fig. 5, we found that G55V, I102T and A219V MC4Rs had significantly increased pERK1/2 levels, similar to that of the WT MC4R (P < 0.05).  $\Delta$ 88-92, L106P and D126Y did not respond to NDP- $\alpha$ -MSH stimulation with ERK1/2 activation.

## Ligand-stimulated activation of ERK1/2 in Class II MC4R mutants

The pERK1/2 levels of 29 Class II mutant MC4Rs (mutants that are translated but are defective in trafficking onto the cell surface), including S58C, N62S, I69R, I69T, P78L, C84R, N97D, G98R, I102S, I125K, Y157S, T162I, R165G, R165W, R165Q, C172R, W174C, I194T, I195S, P260Q, F261S, I269N, C271Y, P299H, L300P, Y302F, A303P, R305Q, and Q307X, were measured after 5 min stimulation with NDP- $\alpha$ -MSH in HEK293T cells transiently transfected with these mutants. As shown in Fig. 6, seven mutants, including I69T, N97D, G98R, I125K, W174C, I194T and F261S, had significantly increased pERK1/2 levels, similar to the WT MC4R (P < 0.05). Eight mutants, including S58C, I195S, P260Q, C271Y, L300P, Y302F, R305Q and Q307X, although responded to NDP- $\alpha$ -MSH stimulation with increased pERK1/2 levels, had lower responses than the WT MC4R. The other fourteen Class II mutants had no response to NDP- $\alpha$ -MSH stimulation in ERK1/2 activation.

## Ligand-stimulated activation of ERK1/2 in Class I MC4R mutants

The pERK1/2 levels of four Class I mutant MC4Rs (mutants that have decreased expression levels), including M79I, S94N, Del170, and C277X, were measured after 5 min stimulation with NDP- $\alpha$ -MSH in HEK293T cells transiently transfected with MC4R mutants. As shown in Fig. 7, we found that M79I and S94N had significantly increased pERK1/2 levels (P < 0.05), whereas Del170 and C277X MC4Rs exhibited impaired ERK1/2 activation.



Figure 6. The ligand-stimulated pERK1/2 levels in HEK293T cells transiently transfected with Class II mutants. Results are expressed as percentage of the value obtained in non-stimulated cells and represent the mean  $\pm$  SEM of five independent experiments. \* indicates significant differences from basal pERK1/2 level (P < 0.05).



Figure 7. The ligand-stimulated pERK1/2 levels in HEK293T cells transiently transfected with Class I mutants. Results are expressed as percentage of the value obtained in non-stimulated cells and represent the mean  $\pm$  SEM of five independent experiments. \* indicates significant differences from basal pERK1/2 level (P < 0.05).



Figure 8. The basal pERK1/2 levels in HEK293T cells transiently transfected with WT or mutant MC4Rs with increased basal pERK1/2 levels (A), or decreased basal pERK1/2 levels (B and C). Results are expressed as percentage of the WT basal pERK1/2 level and represent the mean  $\pm$  SEM of five independent experiments. \* indicates significant differences (P < 0.05).

# Constitutive activities in ERK1/2 signaling in WT and mutant MC4Rs

To evaluate the obesity pathogenesis caused by mutations in the *MC4R* gene through constitutive

MAPK signaling, we compared the basal pERK1/2 levels of these 73 mutants with the WT MC4R. Three mutants, R165G, R165W and C172R, displayed significantly increased basal pERK1/2 signaling (P < 0.05) (Fig. 8A) compared with the basal level of the WT MC4R. The basal pERK1/2 levels of nineteen mutants, including R7C, Y35C, G98R, L106P, D126Y, S136F, H158R, T162I, W174C, M208V, A219V, G231S, G252S, F261S, C277X, L300P, Y302F, R305S and I317V, were significantly decreased compared with that of the WT MC4R (P < 0.05) (Fig. 8B and 8C). The other 51 mutant MC4Rs had similar basal pERK1/2 levels as the WT MC4R (data not shown).

#### Discussion

In addition to the conventional Gs-stimulated adenylyl cyclase pathway, the MC4R also activates MAPKs, especially ERK1/2. The activation of ERK1/2 pathway is one cellular mechanism that may underlie the regulation of energy homeostasis mediated by the MC4R. In this study, we investigated the constitutive and ligand-stimulated activation (pERK1/2) in WT and 73 naturally occurring *MC4R* mutations, including mutations from all five classes. Our hypothesis was that decreased basal or ligand-stimulated ERK1/2 signaling might contribute to obesity pathogenesis caused by mutations in the *MC4R* gene. We were also interested in investigating whether there was biased signaling in these naturally occurring mutations in the Gs-cAMP and ERK1/2 pathway.

In previous functional characterization studies, Class V variants behave similarly as the WT MC4R in heterologous expression systems in all parameters studied, including cell surface expression, ligand binding, and agonist-stimulated cAMP [7, 37]. Whether and how these variants cause energy imbalance and therefore obesity was unclear. We hypothesized that defect in ERK1/2 signaling might contribute to obesity pathogenesis. We measured pERK1/2 levels of 30 Class V variants after 5 min stimulation with NDP-α-MSH. The cell surface expression, ligand binding, and cAMP accumulation (both basal and stimulated) of five variants (C40R, V50M, T112M, A154D, S295P) are similar to the WT MC4R [7, 37, 39, 44]. In the present study, we found that these five variants had impaired ligand-stimulated ERK1/2 activation (Figs. 2 and 3). We propose that defective ERK1/2 signaling in these Class V variants might be a cause of obesity observed in patients harboring these variants.

Class IV mutants are expressed on the cell surface and bind ligand with normal affinity, but are defective in agonist-stimulated signaling (decreased efficacy and/or potency) at the Gs-cAMP pathway, including D90N, S136F, A175T and C326R. The expression of A175T on the plasma membrane is similar to that of the WT receptor, but its ability to generate cAMP in response to ligand is reduced [44, 45]. C326R also has impaired cAMP signaling [46]. Our results indicated that A175T and C326R MC4Rs, although defective in Gs-cAMP signaling, retained normal ERK1/2 signaling in response to NDP- $\alpha$ -MSH stimulation as the WT MC4R, hence exhibiting biased signaling in the Gs-cAMP and ERK1/2 pathways (Fig. 4). In contrast, D90N and S136F MC4Rs, with defective Gs-cAMP signaling, also had decreased ERK1/2 activation, exhibiting no biased signaling (Fig. 4). Interestingly, of all the naturally occurring mutations in the MC4R reported, D90N and S136F are the only mutations that exert dominant negative effect on the WT MC4R [47, 48], very different from the studies in other GPCRs where intracellularly retained GPCRs frequently exert dominant negative effect on the cognate WT receptors when co-expressed [49, 50]. We cannot discern a causal relationship between the lack of biased signaling and dominant negative effect in these two mutations.

Class III mutant MC4Rs are expressed on the cell surface, but are defective in ligand binding *per se*, with either decreased binding capacity and/or affinity, resulting in impairments in hormone stimulated signaling, including G55V,  $\Delta$ 88-92, I102T, L106P, D126Y and A219V. We found that G55V and A219V had increased pERK1/2 levels upon NDP- $\alpha$ -MSH stimulation, although they have significant decrease in binding capacity [40, 43] (Fig. 5), likely due to the presence of spare receptors [49].  $\Delta$ 88-92, L106P and D126Y MC4Rs, with defective ligand binding, also had decreased ERK1/2 activation (Fig. 5).

Class II mutant receptors are produced but are retained intracellularly, most likely in the endoplasmic reticulum due to misfolding being detected by the cell's quality control system [51]. This class comprises the largest set of MC4R mutations reported to date. In this study, we investigated the ERK1/2 signaling of 29 Class II mutants. Our results indicated that seven mutants, I69T, N97D, G98R, I125K, W174C, I194T and F261S, showed increased pERK1/2 levels upon NDP- $\alpha$ -MSH stimulation (Fig. 6). I69T MC4R has only 34% of the cell surface expression and 5% of cAMP signaling of the WT MC4R [52]. N97D and I125K have defective cell surface expression and are unable to generate cAMP in response to ligand stimulation [44, 52], but its MAPK signaling could be stimulated by NDP-a-MSH. G98R has negligible cell surface expression [37]. W174C and F261S have significantly reduced cell surface expression levels compared to the WT MC4R [40]. I194T has decreased cell surface expression, with no measurable ligand binding or signaling to either  $\alpha$ -MSH or  $\beta$ -MSH stimulation [42].

However, these mutant MC4Rs could respond to NDP- $\alpha$ -MSH stimulation with increased ERK1/2 signaling, suggesting that these Class II mutants with partial defect in cell surface expression exhibited biased signaling. In the follicle-stimulating hormone receptor, a naturally occurring mutation results in diminished expression at the cell surface and lack of detectable Gs-cAMP signaling but retains ERK1/2 signaling [53]. In addition, we found that the other 22 Class II mutants had decreased pERK1/2 levels, consistent with the impaired cell surface expression levels in these mutants (Fig. 6).

Due to defective protein synthesis and/or accelerated protein degradation, decreased receptor proteins of Class I mutants are present in the cell. Mutants such as M79I, S94N, Del170, C277X and R305Q likely belong to this class. Although M79I and S94N have relatively normal cell surface expression and ligand binding, they have significantly decreased total expression and partially defective signaling [42]. In the present study, the pERK1/2 levels of M79I and S94N MC4Rs were significantly increased upon NDP- $\alpha$ -MSH stimulation, suggesting that these mutants with partial defect in protein expression could stimulate ERK1/2 signaling (Fig. 7).

Table 1 summarizes the cAMP signaling data previously reported in the literature (together with the original references) and ERK1/2 signaling data reported in this paper. By comparing the two sets of data, we can tentatively identify the biased mutant receptors.

The WT MC4R has some basal activity in cAMP production [54]. Previously, it was suggested that defects in basal cAMP signaling contribute to obesity pathogenesis caused by mutations in the MC4R gene [55]. We asked whether defects in basal MAPK signaling might also cause obesity. We compared the basal pERK1/2 levels of the 73 mutants with the WT MC4R. Nineteen mutants, including R7C, Y35C, G98R, L106P, D126Y, S136F, H158R, T162I, W174C, M208V, A219V, G231S, G252S, F261S, C277X, L300P, Y302F, R305S and I317V, had significantly decreased basal pERK1/2 levels compared with that of the WT MC4R (Fig. 8B,C). We suggest that decreased basal ERK1/2 signaling might contribute to obesity pathogenesis caused by mutations in the MC4R gene, especially for mutants with no defects identified so far, such as Y35C, H158R, G231S, R305S, and I317V. In addition, we found that three mutants, R165G, R165W and C172R, had significantly increased basal pERK1/2 signaling compared with that of the WT MC4R (Fig. 8A). We have observed constitutive activation of ERK1/2 signaling pathway in the MC4R before in both naturally occurring and laboratory-generated mutations [10, 16, 20]. Because the basal activities in the Gs-cAMP pathway for the three mutations, R165G, R165W and C172R, are not increased compared to the WT MC4R [43, 56], but they were constitutively active in the ERK1/2 signaling pathway, therefore they had biased constitutive activity. Previously, in the melanocortin-1 receptor, a mutant receptor constitutively active in the Gs-cAMP pathway is not constitutively active in the ERK1/2 pathway, also representing a case of biased constitutive signaling [57].

R7C       V       1       40]       1       No         T11A       V       1       [7]       1       No         S30F       V       1       [39]       1       No         S30F       V       1       [39]       1       Yes         V37C       V       1       [37]       1       Yes         C40R       V       1       [37]       1       Yes         V48S       V       1       [37]       1       No         V50M       V       1       [37]       1       No         G55V       III       1       [43]       1       Yes         S8C       II       1       [37]       1       No         169R       II       1       [42]       1       No         169T       II       1       [42]       1       No         258C       III       1       [42]       1       No         169T       I       1       [42]       1       No         264R       II       1       [42]       1       No         99DN       IV       1       [47]       1	hMC4R	Class	cAMP	Ref.	pERK1/2	Bias
T11A       V       1       [7]       1       No         S30F       V       1       [56]       1       No         S30F       V       1       [39]       1       Yes         D37V       V       1       [37]       1       Yes         P485       V       1       [37]       1       No         V50M       V       1       [37]       1       No         V50M       V       1       [37]       1       No         655V       II       1       [43]       1       Yes         586C       II       1       [37]       1       No         1697       II       1       [42]       1       No         1698       II       1       [42]       1       No         M791       I       1       [42]       1       No         A892       III       1       [42]       1       No         S94N       I       1       [42]       1       No         970       II       1       [42]       1       No         9755       II       1       [7]       1	R7C	V	↑ (	[40]	1	No
S30FVI[56]INoY35CVI[39]IYesY35CVI[37]IYesC40RVI[37]IYesP48SVI[37]INoC55VIIII[37]INoG55VIIII[37]INoG55VIIII[37]INoG55VIIII[37]INoI69RIII[42]INoI69RIII[22]INoI69RIII[23, 60, 61]INoM79III[41]NoNoM79III[42]NoNoS94NII[42]NoNoS94NII[42]NoNoM02SIII[42]NoNoM10ZII[44]NoNoM10ZIIII[44]NoM10ZIIII[44]NoM112MVI[40]INoM12MVI[40]INoM12MVI[40]INoM12MVI[63]INoM12MVI[63]INoM12MVI[63]INoM12MVI[63]I </td <td>T11A</td> <td>V</td> <td>1</td> <td>[7]</td> <td>↑ 1</td> <td>No</td>	T11A	V	1	[7]	↑ 1	No
Y35C       V       1       [39]       1       No         D37V       V       1       [37]       1       Yes         P48S       V       1       [37]       1       No         V50M       V       1       [37]       1       No         V50M       V       1       [37]       1       No         C55V       III       1       [43]       1       Yes         S86C       II       1       [37], 44, 52]       1       No         169R       II       1       [42]       1       No         169R       II       1       [42]       1       No         169R       II       1       [42]       1       No         A8292       III       1       [42]       1       No         D90N       IV       1       [44]       1       No         S94R       I       1       [42]       1       No         D127       III       1       [44]       1       No         I1028       II       1       [44]       1       No         I1264       III       1       [42]	S30F	V	1	[56]	, ↑	No
D37V       V       1       Yes         C40R       V       1       [37]       1       Yes         P48S       V       1       [37]       1       No         V50M       V       1       [37]       1       Yes         F51L       V       1       [7]       1       No         C55V       III       1       [43]       1       Yes         S88C       II       1       [42]       1       No         I69T       II       1       [42]       1       No         I69R       II       1       [42]       1       No         J79I       I       1       [42]       1       No         J97N       IV       1       [47]       No       No         S44R       II       1       [42]       1       No         J90N       IV       1       [47]       No       No         J90N       II       1       [42]       No       No         J02T       III       1       [42]       No       No         J12M       V       1       [37]       1       Yes	Y35C	V	†	[39]	†	No
C40R       V       1       [39]       1       Yes         P48S       V       1       [37]       1       Yes         P48S       V       1       [37]       1       Yes         S5RC       III       1       [43]       1       Yes         S58C       II       1       [37]       1       No         662S       II       1       [37]       1       No         169R       II       1       [42]       1       No         169R       II       1       [42]       1       No         169T       II       1       [42]       1       No         M791       I       4       [42]       1       No         S88       III       1       [42]       1       No         978L       II       1       [42]       1       No         970D       II       1       [42]       1       No         102S       II       1       [7]       1       No         102S       II       1       [42]       1       No         112M       V       1       [7]       1	D37V	V	†	[37]	i	Yes
PA8S       V       1       [7]       1       No         V50M       V       1       [37]       1       Yes         F51L       V       1       [37]       1       No         G55V       III       1       [43]       1       Yes         S58C       II       1       [37],44,52]       1       No         169R       II       1       [42]       1       No         M79I       I       1       [42]       1       No         S4R       II       1       [42]       1       No         S94N       I       1       [42]       1       No         102T       III       1       [7]       1       No         102S       II       1       [7]       1       No         112M       V       1       [7]       1       No         102F       III       1       [44]       No </td <td>C40R</td> <td>v</td> <td>, ↑</td> <td>[39]</td> <td>Ť</td> <td>Yes</td>	C40R	v	, ↑	[39]	Ť	Yes
X50M       V       1       [27]       1       Yes         F51L       V       1       [37]       1       No         G55V       III       1       [37]       1       No         G55V       III       1       [37]       1       No         G55V       II       1       [37]       1       No         G67       II       1       [52]       1       No         I69R       II       1       [42]       1       No         G78L       II       1       [40]       1       No         M791       I       1       [40]       1       No         G88       II       1       [42]       1       No         990N       IV       1       [7]       1       No         997D       II       1       [42]       1       No         1025       II       1       [7]       1       No         1026P       III       1       [44]       1       No         1124M       V       1       [37]       1       Ne         1125K       II       1       [42]       1 <td>P48S</td> <td>v</td> <td>, ↓</td> <td>[37]</td> <td>÷ ↑</td> <td>No</td>	P48S	v	, ↓	[37]	÷ ↑	No
Total       I       [27]       I       No         C55V       III       [37]       T       No         C55V       II       [37, 44, 52]       I       No         N625       II       [42]       I       No         I69R       II       [42]       I       No         I69T       II       [42]       T       Yes         C84R       II       [42]       T       Yes         C84R       II       [40]       I       No         D90N       IV       [42]       T       Yes         C84R       II       [42]       T       No         D90N       IV       [42]       T       No         S94N       I       T       [42]       No       No         D90N       IV       I       [42]       No       No         D121       II       [42]       No       No       No         D122       III       I       [42]       No       No         D124Y       III       [42]       No       No       No         D126Y       III       I       [37]       No       No	V50M	v	, ↑	[37]	1	Yes
1.11.       V       1 $[43]$ 1       No         C55V       II       1       [37]       1       No         S862       II       1       [37]       1       No         I69R       II       1       [37]       1       No         I69R       II       1       [42]       1       No         I69R       II       1       [42]       1       No         M79I       I       1       [42]       1       No         G8R       II       1       [40]       1       No         S94N       I       1       [42]       1       No         I025       II       1       [7]       1       No         I124       V       1       [37]       1       Yes         I125K       II       1       [42]       1       No         I1264       V       1       [7]       1	F511	v	1	[7]	↓ ↑	No
CDS:       III       1       [43]       1       No         N62S       II       1       [37]       1       No         I69R       II       1       [42]       1       No         I69T       II       1       [52]       1       Yes         P78L       II       1       [42]       1       No         M791       I       1       [42]       1       No         A88-92       III       1       [40]       1       No         S94N       I       1       [42]       1       No         990N       IV       1       [44]       1       No         997D       II       1       [44]       1       No         102S       II       1       [7]       1       No         102T       III       1       [44]       1       No         112K       II       [44, 52]       1       No         112SK       II       [44, 52]       1       No         112SK       II       [44, 52]       1       No         112SK       II       [42]       1       No	C55V	ти III	1	[/3]	 ↑	Voc
3.00.2       II       I       [37, 44, 52]       I       No         169R       II       I       [42]       I       No         169R       II       I       [52]       T       Yes         78L       II       I       [52]       T       Yes         78H       II       I       [40]       I       No         M79I       I       I       [40]       I       No         S48-92       III       I       [47]       I       No         S94N       I       T       [42]       T       No         S97D       II       I       [42]       T       No         1025       II       I       [7]       I       No         1025       II       I       [44]       No       No         112M       V       T       [7]       I       No         1256       II       I       [40] <t< td=""><td>S58C</td><td>п</td><td>↓ ↑</td><td>[37]</td><td>  ↑</td><td>No</td></t<>	S58C	п	↓ ↑	[37]	 ↑	No
NO.5       II       I $[42]$ I       No         I69R       II       I $[42]$ I       No         I69T       II       I $[37, 60, 61]$ I       No         M79I       I       I $[42]$ T       Yes         C84R       II       I $[40]$ I       No         D90N       IV       I $[42]$ T       No         D90N       IV       I $[42]$ T       No         S94N       I       T $[42]$ T       No         D90D       II       I $[42]$ T       No         M02T       III       I $[62]$ T       Yes         I002T       III       I $[7]$ No       No         I124M       V       T $[37]$ Yes       No         S136F       IV       I $[44]$ No       No         S136F       IV       I $[37]$ No       No         T162U       I       I $[52]$ I       No         R165Q       II	N629	11	1	[37] 44 [52]	1	No
109X       II       1       [2]       1       NO         169T       II       1       [52]       f       Yes         P78L       II       1       [42]       f       Yes         C84R       II       1       [40]       1       No         A89-92       III       1       [38]       1       No         D90N       IV       1       [41]       No       No         S94N       I       f       [42]       f       No         997D       II       1       [62]       f       Yes         G98R       II       1       [62]       f       Yes         I025       II       1       [7]       No       No         1021       III       1       [44]       No       No         112M       V       f       [37]       1       No         112M       V       f       [7]       No	1602	11	↓ I	[37, 44, 32]	↓ I	No
1091       1       1 $[52]$ 1       1080         M791       I       1 $[57]$ , 60, 61]       1       No         M791       I       1 $[42]$ †       Yes         C84R       II       1 $[40]$ 1       No         D90N       IV       1 $[47]$ 1       No         S94N       I       1 $[42]$ †       No         S94N       I       1 $[7]$ 1       No         N97D       II       1 $[7]$ 1       No         1025       II       1 $[7]$ 1       No         11021       III       1 $[44]$ 1       No         1124W       V       1 $[37]$ 1       Yes         1125K       II       1 $[42]$ 1       No         S136F       IV       1 $[43]$ 1       No         S145U       V       1 $[53]$ 1       No         S165G       II       1 $[43]$ 1       No         R165Q       II	109K	11	↓ ↓	[42]	↓	INO Mar
P78L       II       I       [42]       1       No $M791$ I       I       [40]       I       No $088$ -92       III       I       [40]       I       No $090N$ IV       I       [47]       I       No $090N$ IV       I       [42]       1       No $097D$ II       I       [42]       1       No $097D$ II       I       [62]       1       Yes $0025$ II       I       [7]       No       No $1027$ III       I       [7]       No       No $11021$ III       [44]       I       No $11024$ V       1       [37]       I       Yes $112M$ V       1       [42]       No       No $5136F$ IV       I       [40]       No       No $1158R$ V       1       [52]       I       No $1158R$ V       1       [52]       No       No $11650$ I       1 <td< td=""><td>1691</td><td>11</td><td>Ļ</td><td>[52]</td><td></td><td>res</td></td<>	1691	11	Ļ	[52]		res
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	P/8L	11	Ļ	[37, 60, 61]	Ļ	NO
C84R       II       I       I40]       I       No         A88-92       III       I       IS8]       I       No         D90N       IV       I       I7       I8       No         S94N       I       1       I42]       1       No         S94N       I       1       I22]       1       No         S97D       II       I       I62]       1       Yes         G98R       II       I       I62]       1       No         I1025       II       I       I7]       No       No         I1027       III       I       I44, 52]       1       No         I112M       V       1       37]       I       No         S136F       IV       I40]       I       No       No         S136F       IV       I       I37]       I       No         H158R       V       1       [63]       1       No         R165W       II       I       I37]       No       No         R165W       II       I       I43]       I       No         R165W       II       I       IA2	M791	1	Ļ	[42]	T	Yes
$\Delta 88-92$ III       I       [38]       I       No         D90N       IV       I       [47]       I       No         S94N       I       1       [42]       1       No         S97D       II       I       [62]       1       Yes         G98R       II       I       [7]       1       No         I102T       III       I       [7]       1       No         I102T       III       I       [7]       1       No         I102T       III       I       [44]       I       No         I112M       V       1       [37]       I       Yes         I125K       II       I       [42]       I       No         S136F       IV       I       [40]       I       No         A154D       V       1       [7]       I       Yes         Y157S       II       I       [37]       I       No         R165G       II       1       [42]       No       No         R165Q       II       I       [42]       I       No         R165Q       II       I       [43] <td>C84R</td> <td>11</td> <td>Ļ</td> <td>[40]</td> <td>Ļ</td> <td>No</td>	C84R	11	Ļ	[40]	Ļ	No
D90N       IV $\downarrow$ [47] $\downarrow$ No         S94N       I $\uparrow$ [42] $\uparrow$ No         S94N       I $\downarrow$ [42] $\uparrow$ No         S97D       II $\downarrow$ [62] $\uparrow$ Yes         G98R       II $\downarrow$ [62] $\uparrow$ Yes         I102T       III $\uparrow$ [7] $\downarrow$ No         I102T       III $\uparrow$ [37] $\downarrow$ Yes         I12M       V $\uparrow$ [37] $\downarrow$ Yes         I12K       II $\downarrow$ [44] $\downarrow$ No         S136F       IV $\downarrow$ [40] $\downarrow$ No         S136F       IV $\downarrow$ [40] $\downarrow$ No         S136F       IV $\downarrow$ [63] $\uparrow$ No         T162I       II $\downarrow$ [52] $\downarrow$ No         T162I       II $\downarrow$ [52, 60, 61] $\downarrow$ No         R165Q       II $\downarrow$ [44] $\uparrow$ Yes	Δ88-92	111	Ļ	[38]	Ļ	No
S94N       I $\uparrow$ No         N97D       II $\downarrow$ [44] $\uparrow$ Yes         G98R       II $\downarrow$ [62] $\uparrow$ Yes         I02S       II $\downarrow$ [7] $\downarrow$ No         I102F       III $\downarrow$ [7] $\downarrow$ No         I102F       III $\downarrow$ [44] $\downarrow$ No         T112M       V $\uparrow$ [37] $\downarrow$ No         S125K       II $\downarrow$ [44] $\downarrow$ No         S125K       II $\downarrow$ [42] $\downarrow$ No         S125K       II $\downarrow$ [42] $\downarrow$ No         S125K       II $\downarrow$ [43] $\downarrow$ No         S136F       IV $\uparrow$ [63] $\uparrow$ No         T162I       II $\downarrow$ [52] $\downarrow$ No         R165G       II $\uparrow$ [43] $\downarrow$ No         R165Q       II $\downarrow$ [44] $\uparrow$ Yes         R165Q       II $\downarrow$ <td>D90N</td> <td>IV</td> <td>Ļ</td> <td>[47]</td> <td>Ļ</td> <td>No</td>	D90N	IV	Ļ	[47]	Ļ	No
N97D       II       I       [44] $\uparrow$ Yes         G98R       II       I       [62] $\uparrow$ Yes         I102S       II       I       [7]       I       No         I102T       III       I       [7]       No       No         I102T       III       I       [44]       I       No         I102H       V $\uparrow$ [37]       I       Yes         I125K       II       I       [44]       I       No         S136F       IV       I       [40]       No       No         A154D       V $\uparrow$ [7]       I       Yes         Y157S       II       I       [37]       I       No         R165Q       II       I       [43]       Yes       No         R165Q       II       I       [42]       No       No         R165Q       II       I       [43]       No       No         R165Q       II       I       [42]       No       No         R165Q       II       I       [42]       No       No         R165Q       II       I	S94N	Ι	↑	[42]	Î	No
G88R       II       I       [62] $\uparrow$ Yes         I102S       II       I       [7]       I       No         I102T       III       I       [7] $\uparrow$ No         I102T       III       I       [44]       No       No         T112M       V $\uparrow$ [37]       I       Yes         I125K       II       I       [44,52] $\uparrow$ Yes         D126Y       III       I       [42]       I       No         S136F       IV       I       [43]       Yes       No         Y157S       II       I       [52, 60, 61]       No       No         R165G       II $\uparrow$ [43]       Yes       No         R165Q       II       I       [42]       No       No         R165Q       II	N97D	II	Ļ	[44]	1	Yes
I102S       II       I       [7]       I       No         I102T       III $\uparrow$ [7] $\uparrow$ No         I106P       III       I       [44]       I       No         I112M       V $\uparrow$ [37]       I       Yes         I125K       II       I       [44,52] $\uparrow$ Yes         D126Y       III       I       [42]       I       No         S136F       IV       I       [40]       I       No         S136F       IV       I       [63] $\uparrow$ No         S157S       II       I       [52]       I       No         R165Q       II $\uparrow$ [43]       I       Yes         R165Q       II       I       [42]       I       No         R165Q       II       I       [42]       I       No         Del170       I       I       [42]       No       No         C172R       II       I       [42]       No       No         Post       I       [42] $\uparrow$ No         M200V       V       [42] $\uparrow$	G98R	II	$\downarrow$	[62]	Î	Yes
I102T       III $\uparrow$ $[7]$ $\uparrow$ No         L106P       III $\downarrow$ [44] $\downarrow$ No         T112M       V $\uparrow$ [37] $\downarrow$ Yes         I125K       II $\downarrow$ [44, 52] $\uparrow$ No         S136F       IV $\downarrow$ [40] $\downarrow$ No         S136F       IV $\downarrow$ [40] $\downarrow$ No         A154D       V $\uparrow$ [7] $\downarrow$ Yes         Y157S       II $\downarrow$ [37] $\downarrow$ No         H158R       V $\uparrow$ [63] $\uparrow$ No         R165G       II $\uparrow$ [43] $\downarrow$ Yes         R165Q       II $\downarrow$ [44, 52, 60] $\downarrow$ No         I170V       V $\uparrow$ [37] $\uparrow$ No         Del170       I       [42] $\downarrow$ No         C172R       II       I       [42] $i$ No         D1955       II $\uparrow$ [42] $\uparrow$ No         D201L	I102S	II	$\downarrow$	[7]	$\downarrow$	No
L106P       III       I       [44]       I       No         T112M       V       1       [37]       I       Yes         1125K       II       I       [44, 52]       1       Yes         D126Y       III       I       [42]       I       No         S136F       IV       I       [40]       I       No         A154D       V       1       [7]       I       Yes         Y157S       II       I       [37]       I       No         H158R       V       1       [63]       1       No         R165G       II       1       [52, 60, 61]       No       No         R165Q       II       1       [44, 52, 60]       No       No         R165Q       II       1       [42]       No       No         R165Q       II       1       [43]       No       No         D170V       V       1       [37]       No       No         C172R       II       443       No       No       No         M200V       V       1       [42]       No       No         M200V       V	I102T	III	↑	[7]	1	No
T112M       V $\uparrow$ [37] $\downarrow$ Yes         I125K       II $\downarrow$ [44, 52] $\uparrow$ Yes         D126Y       III $\downarrow$ [42] $\downarrow$ No         S136F       IV $\downarrow$ [40] $\downarrow$ No         S136F       IV $\uparrow$ [7] $\downarrow$ Yes         Y157S       II $\downarrow$ [37] $\downarrow$ No         H158R       V $\uparrow$ [63] $\uparrow$ No         T162I       II $\downarrow$ [52] $\downarrow$ No         R165G       II $\uparrow$ [43] $\downarrow$ Yes         R165G       II $\downarrow$ [52, 60, 61] $\downarrow$ No         T170V       V $\uparrow$ [37] $\uparrow$ No         Del170       I $\downarrow$ [42] $\downarrow$ No         C172R       II $\downarrow$ [42] $\uparrow$ No         H94T       II $\downarrow$ [42] $\uparrow$ No         H200V       V $\uparrow$ [43] $\uparrow$ No	L106P	III	$\downarrow$	[44]	Ļ	No
I125K       II       I $[44, 52]$ $\uparrow$ Yes         D126Y       III       I $[42]$ $\downarrow$ No         S136F       IV       I $[40]$ $\downarrow$ No         S136F       IV       I $[40]$ $\downarrow$ No         A154D       V $\uparrow$ $[7]$ $\downarrow$ Yes         Y157S       II       I $[37]$ $\downarrow$ No         T162I       II $\downarrow$ $[52]$ $\downarrow$ No         R165G       II $\uparrow$ $[43]$ $\downarrow$ Yes         R165Q       II $\uparrow$ $[43]$ $\downarrow$ No         R165Q       II $\downarrow$ $[42]$ $\downarrow$ No         R165Q       II $\downarrow$ $[42]$ $\downarrow$ No         D170V       V $\uparrow$ $[37]$ $\uparrow$ No         D1717       I $\downarrow$ $[40]$ $\uparrow$ Yes         I170V       V $[42]$ $\uparrow$ No         D1717       I $\downarrow$ $i$ No         I1717       I	T112M	V	↑	[37]	Ļ	Yes
D126Y       III       III       III       III       III       IV       III       IV	I125K	II	Ļ	[44, 52]	1	Yes
S136F       IV       I       [40]       I       No         A154D       V $\uparrow$ [7]       I       Yes         Y157S       II       I       [37]       I       No         H158R       V $\uparrow$ [63] $\uparrow$ No         T162I       II       I       [52]       I       No         R165G       II $\uparrow$ [43]       Yes         R165Q       II       I       [42]       No         R165Q       II       I       [42]       No         R165Q       II       I       [42]       No         R167U       V $\uparrow$ [37] $\uparrow$ No         Del170       I       I       [42]       No       No         C172R       II       I       [43]       No       No         M200V       V $\uparrow$ [42] $\uparrow$ No         M200V       V $\uparrow$ [42] $\uparrow$ No         M200V       V $\uparrow$ [43] $\uparrow$ No         M200V       V $\uparrow$ [43] $\uparrow$ No <td>D126Y</td> <td>III</td> <td>ļ</td> <td>[42]</td> <td>Ļ</td> <td>No</td>	D126Y	III	ļ	[42]	Ļ	No
A154D       V $\uparrow$ $[7]$ $\downarrow$ Yes         Y157S       II $\downarrow$ $[37]$ $\downarrow$ No         H158R       V $\uparrow$ $[63]$ $\uparrow$ No         T162I       II $\downarrow$ $[52]$ $\downarrow$ No         R165G       II $\uparrow$ $[43]$ $\downarrow$ Yes         R165Q       II $\downarrow$ $[44, 52, 60]$ $\downarrow$ No         R165Q       II $\downarrow$ $[42]$ $\downarrow$ No         R165Q       II $\downarrow$ $[43]$ $\downarrow$ No         Del170       I $\downarrow$ $[42]$ $\downarrow$ No         C172R       II $\downarrow$ $[43]$ $\downarrow$ No         M204V       V $\uparrow$ $[42]$ $\uparrow$ No         H95S       II $\uparrow$ $[42]$ $\uparrow$ No         M200V       V $\uparrow$ $[43]$ $\uparrow$ No         M208V       V $\uparrow$ $[43]$ $\uparrow$ No         M218T       V $\uparrow$ $[43]$ $\uparrow$ No	S136F	IV	Ļ	[40]	Ļ	No
Y157S       II       I       [37]       I       No         H158R       V       1       [63]       1       No         T162I       II       I       [52]       I       No         R165G       II       1       [52, 60, 61]       I       No         R165G       II       1       [52, 60, 61]       I       No         R165Q       II       1       [44, 52, 60]       I       No         I170V       V       1       [42]       I       No         Del170       I       I       [42]       No       No         C172R       II       I       [43]       No       No         V174C       II       I       [42]       No       Yes         I194T       II       I       [42]       No       No         M200V       V       1       [7]       No       No         P201L       V       1       [42]       No       No         M208V       V       1       [39]       No       No         M208V       V       1       [43]       No       No         M218T       V	A154D	V	1	[7]	Ì	Yes
H158R       V $\uparrow$ $63$ $\uparrow$ No         T162I       II $\downarrow$ $[52]$ $\downarrow$ No         R165G       II $\uparrow$ $[43]$ $\downarrow$ Yes         R165G       II $\uparrow$ $[43]$ $\downarrow$ Yes         R165Q       II $\downarrow$ $[44, 52, 60]$ $\downarrow$ No         I170V       V $\uparrow$ $[37]$ $\uparrow$ No         Del170       I $\downarrow$ $[42]$ $\downarrow$ No         C172R       II $\downarrow$ $[40]$ $\uparrow$ Yes         A175T       IV $\downarrow$ $[42]$ $\uparrow$ No         M200V       V $\uparrow$ $[42]$ $\uparrow$ No         M200V       V $\uparrow$ $[42]$ $\uparrow$ No         M200V       V $\uparrow$ $[43]$ $\uparrow$ No         M200V       V $\uparrow$ $[43]$ $\uparrow$ No         M208V       V $\uparrow$ $[43]$ $\uparrow$ No         M218T       V $\uparrow$ $[39]$ $\uparrow$ No	Y157S	II	L	[37]	i	No
Ti621       II       I       [52]       I       No         R165G       II       1       [43]       I       Yes         R165G       II       1       [43]       I       Yes         R165Q       II       1       [52, 60, 61]       I       No         R165Q       II       I       [44, 52, 60]       I       No         I170V       V       1       [37]       1       No         Del170       I       I       [43]       I       No         C172R       II       I       [43]       I       No         W174C       II       I       [42]       1       No         V174C       II       I       [42]       1       No         H94T       II       I       [42]       1       No         M200V       V       1       71       No       No         P201L       V       1       [43]       1       No         M208V       V       1       [39]       1       No         M218T       V       1       [39]       1       No         M219V       III       I	H158R	V	* ↑	[63]	Ť.	No
11.1.1       1 $(24)$ $1$ $1$ R165G       II $1$ $[52, 60, 61]$ $\downarrow$ No         R165Q       II $\downarrow$ $[52, 60, 61]$ $\downarrow$ No         R165Q       II $\downarrow$ $[43]$ $\downarrow$ No         R165Q       II $\downarrow$ $[44, 52, 60]$ $\downarrow$ No         Del170       I $\downarrow$ $[42]$ $\downarrow$ No         C172R       II $\downarrow$ $[42]$ $\downarrow$ No         V174C       II $\downarrow$ $[42]$ $\uparrow$ No         V174C       II $\downarrow$ $[42]$ $\uparrow$ No         N174T       II $\downarrow$ $[42]$ $\uparrow$ No         N174C       II $\downarrow$ $[42]$ $\uparrow$ No         N174C       II $\downarrow$ $[42]$ $\uparrow$ No         N200V       V $\uparrow$ $[7]$ $\uparrow$ No         N200V       V $\uparrow$ $[43]$ $\uparrow$ No         M208V       V $\uparrow$ $[43]$ $\uparrow$ No	T162I	п	1	[52]	1	No
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A1731       IV $[44]$ $ $ Yes         I194T       II $[42]$ $\uparrow$ Yes         I195S       II $\uparrow$ $[42]$ $\uparrow$ No         M200V       V $\uparrow$ $[42]$ $\uparrow$ No         F201L       V $\uparrow$ $[43]$ $\uparrow$ No         F202L       V $\uparrow$ $[43]$ $\uparrow$ No         M208V       V $\uparrow$ $[43]$ $\uparrow$ No         M218T       V $\uparrow$ $[39]$ $\uparrow$ No         A219V       III $\downarrow$ $[40]$ $\uparrow$ Yes         G231S       V $\uparrow$ $[64]$ $\uparrow$ No         N240S       V $\uparrow$ $[7]$ $\uparrow$ No         G252S       V $\uparrow$ $[56]$ $\uparrow$ No         Q260Q       II $\downarrow$ $[42]$ $\uparrow$ Yes         P260Q       II $\downarrow$ $[43]$ $\downarrow$ Yes         I269N       II $\uparrow$ $[43]$ $\downarrow$ Yes         I269N	VV174C	11	↓ ↓	[40]	 ↑	Tes Vee
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11955       II $\uparrow$ [42] $\uparrow$ No         M200V       V $\uparrow$ [7] $\uparrow$ No         F201L       V $\uparrow$ [42] $\uparrow$ No         F202L       V $\uparrow$ [43] $\uparrow$ No         M208V       V $\uparrow$ [43] $\uparrow$ No         M218T       V $\uparrow$ [39] $\uparrow$ No         A219V       III       [40] $\uparrow$ Yes         G231S       V $\uparrow$ [64] $\uparrow$ No         A219V       III       [42] $\uparrow$ No         G231V       V $\uparrow$ [64] $\uparrow$ No         G231V       V $\uparrow$ [42] $\uparrow$ No         R2405       V $\uparrow$ [7] $\uparrow$ No         P260Q       II $\downarrow$ [42] $\uparrow$ Yes         P260Q       II $\downarrow$ [43] $\downarrow$ Yes         C269N       II $\downarrow$ [43] $\downarrow$ Yes         C271Y       II $\downarrow$	11941	11	Ļ	[42]	1	Yes
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F202L       V $\uparrow$ [43] $\uparrow$ No         M208V       V $\uparrow$ [43] $\uparrow$ No         M218T       V $\uparrow$ [39] $\uparrow$ No         A219V       III $\downarrow$ [40] $\uparrow$ Yes         G231S       V $\uparrow$ [64] $\uparrow$ No         G231V       V $\uparrow$ [64] $\uparrow$ No         G231V       V $\uparrow$ [64] $\uparrow$ No         G231V       V $\uparrow$ [65] $\uparrow$ No         G252S       V $\uparrow$ [56] $\uparrow$ No         P260Q       II $\downarrow$ [42] $\uparrow$ Yes         F261S       II $\downarrow$ [40] $\uparrow$ Yes         I269N       II $\uparrow$ [43] $\downarrow$ Yes         C271Y       II $\downarrow$ [37, 44, 52] $\uparrow$ Yes         No       [65] $i$ No       No	F201L	V	Î	[42]	Î	No
M208V       V $\uparrow$ [43] $\uparrow$ No         M218T       V $\uparrow$ [39] $\uparrow$ No         A219V       III $\downarrow$ [40] $\uparrow$ Yes         G231S       V $\uparrow$ [64] $\uparrow$ No         C231V       V $\uparrow$ [42] $\uparrow$ No         N240S       V $\uparrow$ [7] $\uparrow$ No         C252S       V $\uparrow$ [56] $\uparrow$ No         C252S       V $\uparrow$ [56] $\uparrow$ No         P260Q       II $\downarrow$ [42] $\uparrow$ Yes         F261S       II $\downarrow$ [43] $\downarrow$ Yes         C271Y       II $\uparrow$ [37] $\downarrow$ Yes         N274S       V $\uparrow$ [37] $\downarrow$ Yes         C277X       I $\downarrow$ [65] $\downarrow$ No	F202L	V	Î	[43]	Î	No
M218T       V $\uparrow$ [39] $\uparrow$ No         A219V       III $\downarrow$ [40] $\uparrow$ Yes         G231S       V $\uparrow$ [64] $\uparrow$ No         G231V       V $\uparrow$ [42] $\uparrow$ No         N2405       V $\uparrow$ [56] $\uparrow$ No         N22602       II $\downarrow$ [42] $\uparrow$ Yes         P260Q       II $\downarrow$ [42] $\uparrow$ Yes         F261S       II $\downarrow$ [43] $\downarrow$ Yes         C271Y       II $\downarrow$ [37] $\downarrow$ Yes         C277X       I       I       [65]       I       No	M208V	V	↑	[43]	Î	No
A219VIII $\downarrow$ $[40]$ $\uparrow$ YesG231SV $\uparrow$ $[64]$ $\uparrow$ NoG231VV $\uparrow$ $[64]$ $\uparrow$ NoN2405V $\uparrow$ $[7]$ $\uparrow$ NoC2525V $\uparrow$ $[56]$ $\uparrow$ NoP260QII $\downarrow$ $[42]$ $\uparrow$ YesF261SII $\downarrow$ $[40]$ $\uparrow$ YesL269NII $\uparrow$ $[43]$ $\downarrow$ YesC271YII $\downarrow$ $[37]$ $\downarrow$ YesC277XI $\downarrow$ $[56]$ $\downarrow$ No	M218T	V	↑	[39]	Î	No
G231SV $\uparrow$ [64] $\uparrow$ NoG231VV $\uparrow$ [42] $\uparrow$ NoN240SV $\uparrow$ [7] $\uparrow$ NoG252SV $\uparrow$ [56] $\uparrow$ NoP260QII $\downarrow$ [42] $\uparrow$ YesF261SII $\downarrow$ [40] $\uparrow$ YesC271YII $\downarrow$ [37] $\downarrow$ YesC277XI $\downarrow$ [65] $\downarrow$ No	A219V	III	Ļ	[40]	1	Yes
G231VV $\uparrow$ [42] $\uparrow$ NoN2405V $\uparrow$ [7] $\uparrow$ NoG2525V $\uparrow$ [56] $\uparrow$ NoP260QII $\downarrow$ [42] $\uparrow$ YesF261SII $\downarrow$ [40] $\uparrow$ YesI269NII $\uparrow$ [43] $\downarrow$ YesC271YII $\downarrow$ [37, 44, 52] $\uparrow$ YesN2745V $\uparrow$ [37] $\downarrow$ YesC277XI $\downarrow$ [65] $\downarrow$ No	G231S	V	1	[64]	Î	No
N2405V $\uparrow$ $[7]$ $\uparrow$ NoG2525V $\uparrow$ $[56]$ $\uparrow$ NoP260QII $\downarrow$ $[42]$ $\uparrow$ YesF2615II $\downarrow$ $[40]$ $\uparrow$ YesI269NII $\uparrow$ $[43]$ $\downarrow$ YesC271YII $\downarrow$ $[37, 44, 52]$ $\uparrow$ YesN2745V $\uparrow$ $[37]$ $\downarrow$ YesC277XI $\downarrow$ $[65]$ $\downarrow$ No	G231V	V	1	[42]	Î	No
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	N240S	V	↑	[7]	1	No
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	G252S	V	<b>↑</b>	[56]	↑	No
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	P260Q	II	Ļ	[42]	↑	Yes
I269NII $\uparrow$ [43] $\downarrow$ YesC271YII $\downarrow$ [37, 44, 52] $\uparrow$ YesN274SV $\uparrow$ [37] $\downarrow$ YesC277XI $\downarrow$ [65] $\downarrow$ No	F261S	II	Ļ	[40]	↑	Yes
C271Y     II     ↓     [37, 44, 52]     ↑     Yes       N274S     V     ↑     [37]     ↓     Yes       C277X     I     ↓     [65]     ↓     No	I269N	II	Î	[43]	Ļ	Yes
N274S V ↑ [37] ↓ Yes C277X I ↓ [65] ↓ No	C271Y	II	Ļ	[37, 44, 52]	1	Yes
C277X I [65] No	N274S	V	↑	[37]	Ļ	Yes
· · · · · · · · · · · · · · · · · · ·	C277X	Ι	Ļ	[65]	Ļ	No

I289L	V	Î	[42]	↑	No
S295P	V	Î	[7]	$\downarrow$	Yes
P299H	Π	Ļ	[66]	$\downarrow$	No
L300P	Π	Î	[42]	↑	No
Y302F	II	Ť	[41]	↑	No
A303P	II	Ť	[43]	$\downarrow$	Yes
R305Q	II	Ť	[46]	↑	No
R305S	V	Ť	[42]	↑	No
Q307X	Π	Ļ	[42]	↑	Yes
I317V	V	Î	[40]	↑	No
L325F	V	Ť	[40]	↑	No
C326R	IV	$\downarrow$	[46]	↑	Yes
Y332C	V	Ť	[42]	↑	No
Y332H	V	Ť	[42]	↑	No

"↑": denotes normal ligand-stimulated response as WT MC4R. "↓": denotes defective ligand-stimulated response compared to WT MC4R. "Bias" column is designed to demonstrate whether ligand-stimulated cAMP and pERK1/2 signaling pathways were divergent. "Yes" denotes biased activation of either signaling pathway; "No" denotes balanced cAMP and pERK1/2 signaling.

Although this is the first comprehensive study on biased signaling in naturally occurring mutations in the MC4R, these findings need to be further confirmed in neuronal cells since the MC4R is expressed primarily in the central nervous system in vivo. Our recent studies on the pharmacoperones for the MC4R compared the rescuing efficacy in HEK293 cells and neuronal cells [58, 59]. Our results showed that although the results are very similar, a few differences indeed are observed. More importantly, future studies need to confirm the in vivo relevance of mutant receptors with biased signaling properties in regulating energy homeostasis using knockin mice. For the MC4R, proximal (conformational changes in the receptor upon binding of biased ligand or receptor mutation), intermediate (from conformational change into downstream signaling), and distal (effects of differential signaling on the gene expression and physiology) mechanisms of biased signaling [23] all remain to be further explored.

In conclusion, the results of the present study demonstrated that nineteen mutants had significantly decreased basal pERK1/2 level, and five Class V variants had impaired ligand-stimulated ERK1/2 activation. The decreased basal or ligand-stimulated ERK1/2 signaling might contribute to obesity pathogenesis caused by mutations in the *MC4R* gene. We also observed biased signaling in these naturally occurring mutations in the Gs-cAMP and ERK1/2 pathway.

## Abbreviations

ERK1/2: extracellular signal-regulated kinases 1 and 2; GPCR: G protein-coupled receptor; MAPK: mitogen-activated protein kinas; MC4R: melanocortin-4 receptor; NDP- $\alpha$ -MSH: [Nle<sup>4</sup>,D-Phe<sup>7</sup>]- $\alpha$ melanocyte stimulating hormone; pERK1/2: phosphorylated ERK1/2; WT: wild type.

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## **Competing Interests**

The authors have declared that no competing interest exists.

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