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# Functional characterization of novel melanocortin-3 receptor mutations identified from obese subjects

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**Abstract**

It is controversial whether mutation in the melancortin-3 receptor (MC3R) gene is a cause for monogenic obesity in humans. Three novel mutations in the *MC3R*, A293T, I335S, and X361S, were identified from morbidly obese subjects. We investigated whether these mutations caused loss-of-function and the molecular defects if any. Ligand binding, signaling, and cell surface expression of the mutant MC3Rs were studied. I335S resulted in a complete loss of ligand binding and signaling due to intracellular retention. A293T and X361S MC3Rs had normal ligand binding and signaling as wild type MC3R. Co-expression studies showed that the mutants did not affect wild type MC3R signaling. Hence the I335S variant previously identified from obese patients is not expressed at the cell surface when expressed in vitro, suggesting that it might contribute to obesity in carriers of this variant. Whether A293T and X361S cause obesity remains to be investigated. Additional mutations at I335 showed that I335, part of the highly conserved N/DPxxY motif, was critical for multiple aspects of the MC3R function, including cell surface expression, ligand binding, and signaling.


*Keywords:* Melanocortin-3 receptor; Mutation; Obesity; G protein-coupled receptor; Trafficking

## 1. Introduction

Two neural melanocortin receptors (MCRs), the melanocortin-3 and -4 receptors (MC3R and MC4R), have been cloned [1–3]. These receptors are G protein-coupled receptors (GPCRs) that couple primarily to the stimulatory heterotrimeric GTP-binding protein Gs. Therefore, ligand binding to these receptors results in increased cAMP level in the cell.

Mouse genetic studies revealed non-redundant roles of these two MCRs in energy homeostasis. Whereas the MC4R regulates food intake as well as energy expenditure, the MC3R does not affect food intake [4–6]. Through mechanism(s) still not completely understood, the MC3R regulates feeding efficiency and fat deposition. Mice lacking both MC3R and MC4R have exacerbated obesity compared with MC3R or MC4R single gene knockout mice, again suggesting that the MC3R and MC4R serve non-redundant roles in energy homeostasis [5]. The two original knockout studies were done in C57BL/6J mice, a mouse line prone to obesity [5,6]. A recent study showed that in a mouse strain that is responsive to leptin and resistant to obesity (Black Swiss;129), MC3R knockout mice had a comparable degree of increased adiposity as the MC4R knockout mice [7], emphasizing the importance of MC3R in energy homeostasis.

Earlier human genetic studies did not identify any mutations in *MC3R* from patients with obesity and Type 2 diabetes mellitus [8–11] despite strong linkage of the loci encompassing *MC3R* on chromosome 20q.13 with these traits [12–16]. In 2002, a potential mutation (I183N) was reported from two obese patients [17]. Functional analysis showed that this mutation indeed resulted in loss-of-function [18–20]. We showed that this mutation, which changed a highly conserved Ile/Val at the end of transmembrane domain (TM) 3, resulted in a defect in G protein-coupling/activation. The mutant receptor bound to the ligand (therefore it was expressed on the cell surface) but could not convey this binding to increased cAMP generation [18]. These

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functional analyses together with the original clinical findings are strongly supportive of a role of the MC3R in regulating energy homeostasis in humans; a defect in this regulation results in obesity. However, I183N was the only mutation reported. Recently, DiBlasio and her colleagues screened 110 unrelated morbidly obese subjects with a mean body mass index (BMI) of 47.2 kg/m$^2$ [21]. Another selection criteria for these subjects was that they had resting energy expenditure/food intake ratios of greater than 0.7, indicating that they were obese despite burning more than 70% of their food intake [21]. Three novel *MC3R* variants, A293T, I335S, and X361S, were identified. Subjects harboring A293T or I335S variants had increased fat mass and decreased lean mass. Their leptin levels were lower than expected for their BMI. The subject with the X361S variant, a 22 years old woman with a BMI of 44.3 kg/m$^2$, had a very high amount of fat mass (53.8%) and high serum leptin levels [21]. Family studies with two variants showed co-segregation of the variant with obesity: heterozygotes for the variants were all obese. In addition, these three genetic variants were not found in 100 normal weight subjects [21]. However, the functional properties of these variants were unknown. We sought to characterize the functional properties of these variants in vitro.

## 2. Materials and methods

### 2.1. Hormones and supplies

[Nle$^4$,D-Phe$^7$]-α-MSH (NDP-MSH) was purchased from Bachem (King of Prussia, PA). Iodinated NDP-MSH was purchased from the Peptide Radioiodination Service Center at the University of Mississippi (University, MS). Tissue culture plastic wares were purchased from Corning (Corning, NY). Cell culture media, newborn calf serum, and other reagents for cell culture were obtained from Invitrogen (Carlsbad, CA).

### 2.2. In vitro mutagenesis of the MC3R

The wild type (WT) MC3R tagged at the N-terminus with 3xHA tag was obtained from UMR cDNA Resource Center (www.cDNA.org). Mutations were introduced into WT MC3R by QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) as detailed before [22]. The primers used for generating the naturally occurring mutants are listed in Table 1. For the X361S mutant, the original stop codon was replaced by AGC (coding for serine) followed by GATGCAGGGCCATGGAAA (coding for DAGPWK) followed by the new stop codon. The nucleotide sequence used for coding DAGPWK was the same as in the genomic sequence of the human MC3R. Qiagen Maxiprep kit (Qiagen, Valencia, CA) was used to prepare the plasmids for transfection. Automated DNA sequencing was used to confirm that only the intended mutations were introduced in the constructs.

Table 1
Primers used to construct the naturally occurring MC3R mutants

| Mutant | Forward oligonucleotide primer |
|---|---|
| A293T | CATCTTCTGCTGG**ACC**CCCTTCTTCC |
| I335S | CATCGACCCACTC**TCC**TACGCTTTCCGG |
| X361S | GGCATGAACTTGGGA***AGC*GATGCAGGGCCATGGAAA***TGA***-GATCCACTAGTCCAG |

The nucleotides introducing the mutations are highlighted in bold. For the mutant X361S, the original stop codon was replaced by AGC coding for serine (highlighted in both bold and italic), followed by nucleotides coding for DAGPWK as found in the genomic sequence (highlighted in bold) and the new stop codon (in italic).

### 2.3. Cells and transfections

HEK293 cells, obtained from American Type Culture Collection (Manassas, VA), were maintained at 5% $CO_2$ in Dulbecco's modified Eagle's medium containing 50 μg/ml gentamicin, 10 mM HEPES, and 10% newborn calf serum. Cells were plated on gelatin-coated 35 mm 6-well clusters. When the cells reached 50–70% confluency, they were transfected using the calcium precipitation method [23]. Four μg plasmid in 2 ml media was used per 35 mm dish. Cells were used approximately 48 h after transfection for measuring ligand binding and signaling. In co-expression studies, 2 μg plasmid of WT and mutant were co-transfected, whereas 2 μg empty vector was added to the WT group so that all groups received the same amount of DNA.

### 2.4. Ligand binding to intact cells

The methods for ligand binding were described in detail before [22]. Briefly, 48 h after transfection, cells were washed twice with warm Waymouth/BSA (Waymouth's MB752/1 media containing 1 mg/ml bovine serum albumin (BSA)) (herein referred as Waymouth/BSA) and incubated with 100,000 cpm of $^{125}$I-NDP-MSH with or without different concentrations of unlabeled NDP-MSH (from $10^{-12}$ to $10^{-6}$ M) at 37 C for 1 h. Cells were then placed directly on ice, washed twice with cold Hank's balanced salt solution containing 1 mg/ml BSA and 50 μg/ml gentamicin. Then 100 μl of 0.5 N NaOH was added to each well. Cell lysates were then collected using cotton swabs, and counted in gamma counter. Binding capacity and $IC_{50}$ values were calculated using GraphPad Prism 4.0 software (San Diego, CA).

### 2.5. Ligand stimulation of intracellular cAMP production

HEK293 cells were plated and transfected as described above. Approximately 48 h after transfection, cells were washed twice with warm Waymouth/BSA. Then 1 ml of fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma) was added to each well. After 15 min incubation at 37 C, either buffer alone or different concentrations of NDP-MSH were added and the incubation was continued for another hour. The cells were then placed on ice, media were aspirated, and intracellular cAMP was extracted by the addition of 0.5 N percholoric acid containing 180 μg/ml theophylline, and measured using radioimmunoassay. All determinations were performed in triplicate. Iodinated cAMP was prepared using chloramine T method. Maximal responses ($R_{max}$) and $EC_{50}$ values were calculated using Prism 4.0 software.

### 2.6. Localization of MC3R expression by confocal microscopy

HEK293 cells plated onto poly-D-lysine-coated slides (Biocoat cellware from Falcon) were transiently transfected with WT or mutant MC3Rs tagged at the N terminus with three tandem HA epitope tags and immunostained with fluorescein-conjugated anti-HA antibody. For immunohistochemistry, all solutions and procedures were at room temperature. On the day of the experiment, 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. After a further wash of three times with PBS-IH, cells were permeabilized with 1% Triton X-100 in PBS-IH for 5 min. For cell surface staining, this step was omitted. Then the cells were incubated with blocking solution (5% BSA in PBS-IH) for 1 h, and incubated 1 h with fluorescein-conjugated anti-HA.11 antibody (Covance, Berkeley, CA) diluted 1:100 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 Zeiss LSM510 laser scanning confocal microscope with excitation by 488 nm argon laser and detected with a 530- to 560-nm filter.

### 2.7. Statistical analyses

Student's *t* test was used to determine the significance of differences in the signaling and binding parameters between wt and mutant MC3Rs. The statistical analysis was carried out using GraphPad Prism 4.0.

## 3. Results

Recently, three novel *MC3R* variants were identified from morbidly obese patients in Italy: A293T in TM6, I335S in TM7, and X361S [21]. X361S changed the stop codon to Ser resulting in the addition of seven amino acids before the downstream stop codon, so the C terminus starting with the original stop codon is: $^{361}$SDAGPWK. However, the functional properties of these mutants were unknown. In this study, we sought to determine whether these mutations resulted in loss-of-function and what were the functional defects. NDP-MSH, a superpotent agonist of α-MSH [24], was used for ligand binding and signaling studies.

### 3.1. Signaling properties of the mutant receptors

First, we tested whether the three MC3R variants could respond to NDP-MSH stimulation with increased cAMP generation. As shown in Fig. 1A, I335S MC3R was totally devoid of signaling in response to NDP-MSH stimulation. A293T and X361S signaled relatively normally to NDP-MSH stimulation with similar $R_{max}$ as WT MC3R. The $EC_{50}$ for A293T was similar to WT MC3R, whereas that of the X361S was slightly increased (Table 2). In contrast to the MC4R, the WT MC3R had no constitutive activity. The basal cAMP levels in cells transfected with WT MC3R were similar to the basal cAMP levels in cells transfected with empty vector pcDNA3. The MC3R variants did not cause any change in constitutive activity compared to WT MC3R (data not shown).

### 3.2. Ligand binding properties of the mutant receptors

To investigate whether the mutant MC3Rs had normal binding to the ligands, we performed competitive binding using $^{125}$I-NDP-MSH as the iodinated ligand and cold NDP-MSH as the competitor. Fig. 1B showed results from a typical experiment. I335S did not have any specific binding to NDP-MSH, whereas A293T and X351S had similar binding capacities and $IC_{50}$s as the WT MC3R (Table 2).

Table 2
Ligand binding and agonist-stimulated cAMP production of WT and mutant MC3Rs

| MC3R | *n* | NDP-MSH binding | | NDP-MSH-stimulated cAMP | |
|---|---|---|---|---|---|
| | | $IC_{50}$ (nM) | $B_{max}$ (% wt) | $EC_{50}$ (nM) | $R_{max}$ (% wt) |
| WT | 10 | 6.83±2.55 | 100 | 0.67±0.17 | 100 |
| A293T | 5 | 7.18±1.26 | 98±10 | 1.02±0.47 | 89±12 |
| I335S | 5 | ND | ND | ND | ND |
| X361S | 5 | 6.35±3.37 | 104±8 | 2.43±1.09 | 98±22 |
| WT + A293T | 4 | 4.11±1.05 | 106±9 | 0.88±0.39 | 98±22 |
| WT + I335S | 4 | 2.18±0.06** | 90±5 | 0.51±0.09 | 69±14 |
| WT + X361S | 4 | 3.76±2.06 | 94±10 | 0.46±0.22 | 85±24 |
| I335A | 4 | ND | ND | ND | ND |
| I335D | 4 | ND | ND | ND | ND |
| I335L | 4 | 0.58±0.10*** | 4±1*** | 0.57±0.22*** | 4±1*** |
| I335N | 4 | ND | ND | ND | ND |
| I335R | 4 | ND | ND | ND | ND |
| I335V | 4 | 2.94±0.92 | 100±6 | 0.43±0.05 | 44±9** |

Data shown are the mean±SEM of the indicated number of experiments. The $B_{max}$ of cells expressing WT MC3R was 0.047±0.012 nmol NDP-MSH bound/$10^6$ cells and the $R_{max}$ was 1328.0±174.5 pmol cAMP/$10^6$ cells (mean±SEM of 10 experiments).
ND: not detectable.
**: $p<0.01$ compared with WT MC3R.
***: $p<0.001$ compared with WT MC3R.

### 3.3. Localization of I335S MC3R by confocal microscopy

To investigate whether I335S was defective in ligand binding *per se* or was defective in trafficking to plasma membrane, we performed confocal laser scanning microscopy studies using MC3Rs tagged at the N-terminus with 3 tandem HA epitope

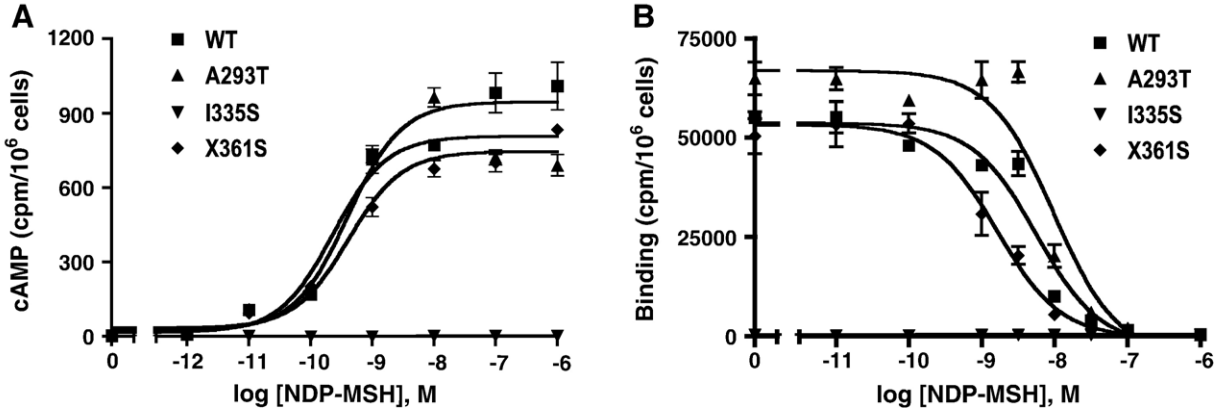


Fig. 1. Accumulation of intracellular cAMP and competitive binding of iodinated NDP-MSH in cells transiently transfected with WT and mutant MC3Rs. Panel A: HEK293 cells were transiently transfected with WT or mutant MC3Rs, stimulated with different concentrations of NDP-MSH, and intracellular cAMP levels measured with RIA. Results are expressed as mean±SEM of triplicate determinations within one experiment. All experiments were performed five times and similar results were obtained. Panel B: HEK293 cells were transiently transfected with WT or mutant MC3Rs and ligand binding assays were performed as described in Materials and methods. Different concentrations of unlabeled NDP-MSH were used to inhibit the binding of $^{125}$I-NDP-MSH to WT and mutant MC3Rs. Results shown are mean± range of duplicate determinations within one experiment. All experiments were performed five times and similar results were obtained.

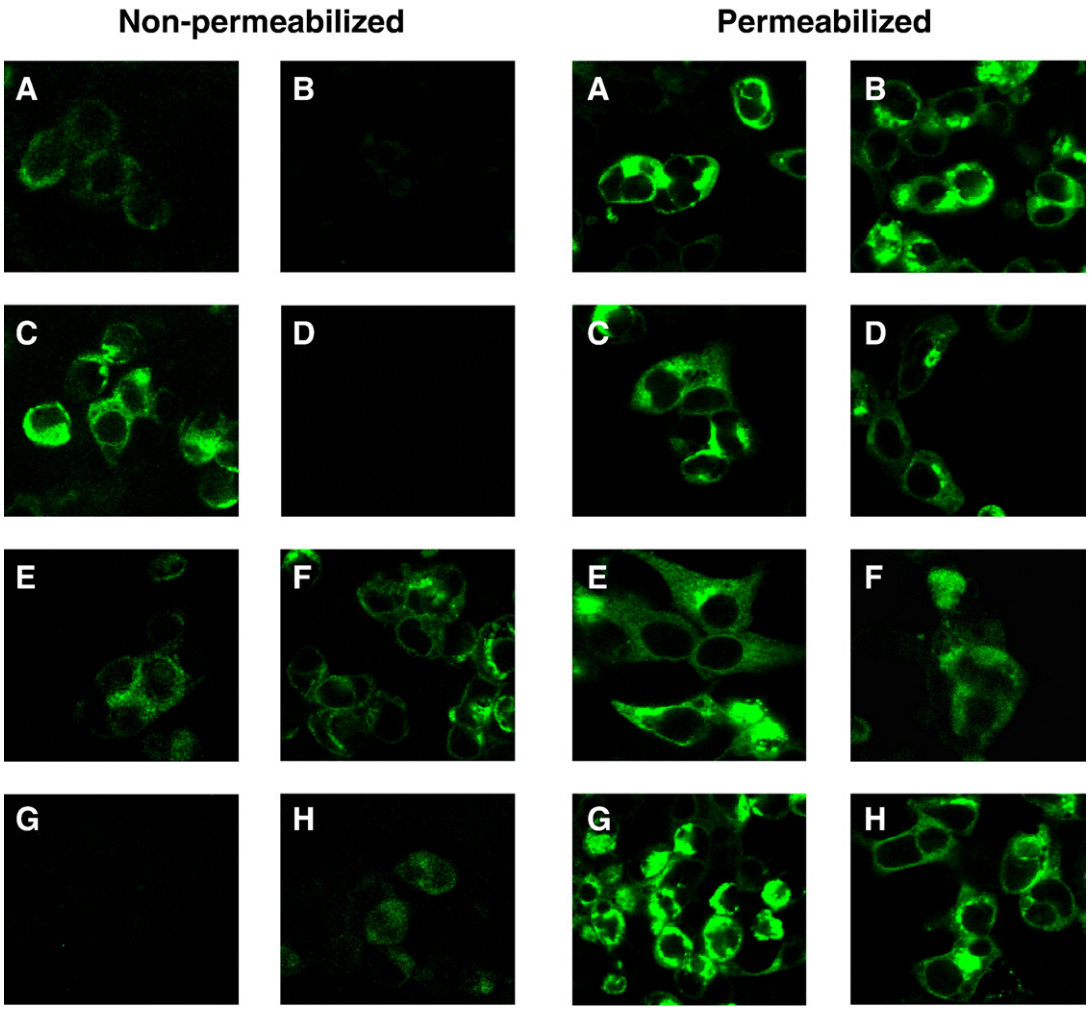


Fig. 2. Confocal imaging of WT and mutant MC3Rs. HEK293 cells expressing WT and mutant MC3Rs were permeabilized or left intact as indicated and then stained with fluorescein-conjugated anti-HA monoclonal antibody and imaged by confocal microscopy. Panels are: A, WT; B: I335S; C, I335A; D, I335D; E, I335L; F, I335N; G, I335R; H, I335V.

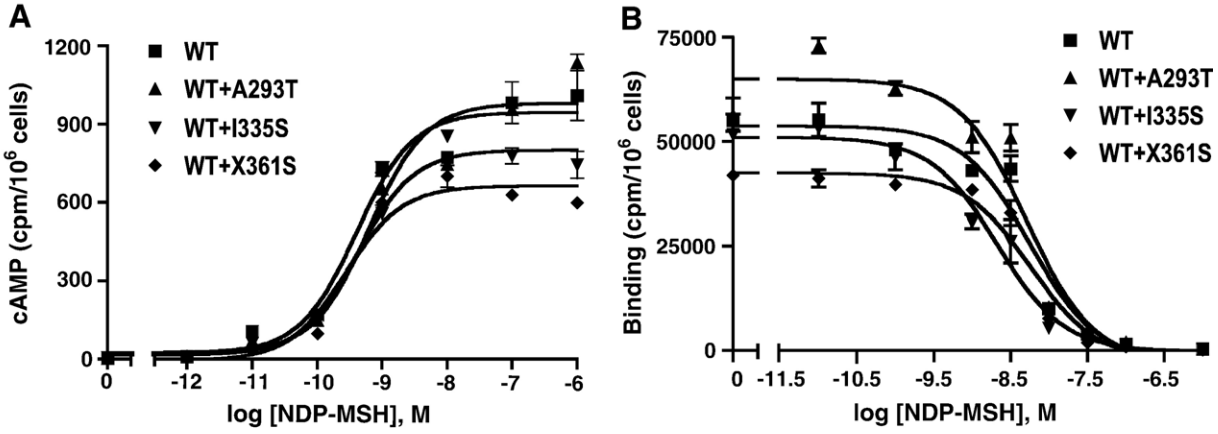


Fig. 3. Accumulation of intracellular cAMP and agonist binding in cells expressing WT and/or mutant MC3Rs. HEK293 cells were transiently transfected with the indicated MC3R constructs, and intracellular cAMP accumulation and binding assays were performed. The same amount of WT MC3R plasmid was present in all groups. Empty vector was added to keep plasmid concentrations constant among the different groups. Panel A: HEK293 cells transiently transfected with the MC3R constructs were stimulated with various concentrations of NDP-MSH and intracellular cAMP levels were measured. Results are expressed as the mean±SEM of triplicate determinations within one experiment. All experiments were performed four times and similar results were obtained. Panel B: HEK293 cells transiently transfected with the MC3R constructs were used to measure ligand-binding properties. Different concentrations of unlabeled NDP-MSH were used to displace the binding of [$^{125}$I]-NDP-MSH to WT or mutant MC3Rs on intact cells. Results shown are expressed as the mean±range of duplicate determinations within one experiment. All experiments were performed four times and similar results were obtained.

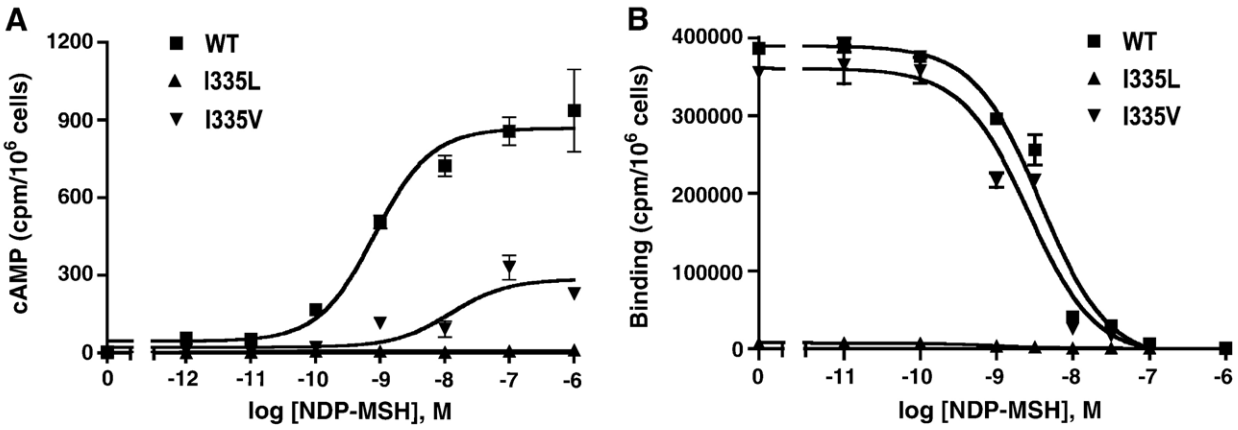


Fig. 4. Multiple mutagenesis of I335. I335 was mutated to Ala, Asn, Asp, Arg, Leu, Val, and tested for their signaling (A) and ligand binding (B) properties. Panel A: HEK293 cells transiently transfected with WT or mutant MC3Rs were stimulated with different concentrations of NDP-MSH. Intracellular cAMP levels were measured with RIA. Results are expressed as mean ± SEM of triplicate determinations within one experiment. The experiments were performed four times and similar results were obtained. Panel B: HEK293 cells were transiently transfected with WT or mutant MC3Rs and ligand binding assays were performed. Different concentrations of unlabeled NDP-MSH were used to compete the binding of $^{125}$I-NDP-MSH to WT and mutant MC3Rs. Results shown are mean ± range of duplicate determinations within one experiment. All experiments were performed four times and similar results were obtained. There were no signaling or ligand binding for I335A, I335N, I335D, and I335R (data not shown).

tags. Preliminary studies showed that the addition of the epitope tag did not affect ligand binding or signaling significantly (data not shown). Cells transiently transfected with WT or I335S MC3Rs were stained with fluorescein-conjugated anti-HA antibody and visualized under a confocal microscope. As shown in Fig. 2, WT MC3R was expressed on the cell surface as well as intracellularly. I335S was devoid of cell surface staining, whereas permeabilization revealed intracellular staining (Fig. 2) suggesting that the mutant receptor was expressed but retained intracellularly.

### *3.4. Co-expression of WT and mutant MC3Rs*

To investigate whether the mutant MC3Rs had any dominant negative activity on WT MC3R, co-expression experiments were performed. Cells were co-transfected with WT and mutant MC3Rs. Empty vector was used so that all groups had the same amount of plasmids transfected. The results of these experiments showed that the mutant MC3Rs had no dominant negative activity on the WT MC3R signaling or binding (Fig. 3A and B and Table 1). In fact, cells co-transfected with I335S had significantly decreased $IC_{50}$ (increased binding affinity) (Table 2).

### *3.5. Multiple mutagenesis of I335 revealed its critical importance in MC3R function*

Isoleucine at codon 335 belongs to the highly conserved N/DPxxY motif (Asn/Asp–Pro–Xaa–Xaa–Tyr) in TM7. In the MC3R, it is DPLIY. To gain further insight into the importance of I335 in cell surface expression of the MC3R, we generated multiple mutants that changed I335 to hydrophobic (Ala, Leu, and Val), polar (Asn), or charged (Asp and Arg) residues. The results of these experiments showed that mutants I335A, I335D, I335N, and I335R did not exhibit any binding to $^{125}$NDP-MSH or signaling in response to NDP-MSH stimulation (Table 2 and data not shown). I335L retained minimal although discernible binding and signaling. I335V had similar binding properties as WT MC3R; however, the maximal response decreased by half (Fig. 4 and Table 2). The $EC_{50}$s for I335L and I335V were similar to that of the WT MC3R (Table 2).

Confocal microscopy experiments showed that mutants that changed Ile into charged residues (I335D and I335R) were expressed but totally retained intracellularly. The other mutants, including I335A, I335N, I335L, and I335V, could be detected in non-permeabilized cells, suggesting that they were expressed on the cell surface (Fig. 2). These results suggested that I335 was important in MC3R function. When mutated, it could result in intracellular retention or defects in ligand binding and signaling depending on the amino acids introduced.

## 4. Discussion

In this study, we characterized three novel naturally occurring *MC3R* variants recently identified from morbidly obese subjects [21]. We showed that I335S was totally devoid of ligand binding and signaling due to intracellular retention (Figs. 1, 2), suggesting that I335S might contribute to obesity in the patients harboring the variant allele. Previous studies in other GPCRs have shown that intracellular retention is the most common defect of naturally occurring mutations in GPCRs that cause human diseases (reviewed in [22,25,26]). Especially relevant is that intracellular retention is the most common defect in MC4R mutations associated with obesity [22,27–29]. Recently, Beaumont and colleagues showed that some of the melanocortin-1 receptor mutations associated with pigmentation defects also had reduced cell surface expression [30].

Analysis of the polymorphic variant T6K/V81I showed that it had reduced total protein level. However, the localization of the receptor was not altered [31]. The two novel variants identified in Singaporean patients (A70T and M134I) were reported to have defects in signaling [20].

The other two MC3R variants, A293T and X361S, did not cause major defects in cell surface expression, ligand binding and signaling (Fig. 1 and Table 2). When co-expressed with WT MC3R, they also did not affect WT MC3R signaling (Fig. 3). Whether these two mutants were the cause of morbid obesity observed in the patients remain unknown. In addition, the clinical and genetic data on the identification of these mutations are eagerly awaited in peer-reviewed literature.

Although it is well established that mutations in the *MC4R* is the most common monogenic form of obesity, it is controversial whether mutations in the *MC3R* can also cause human obesity. Previously, several large scale screening studies did not identify any mutations in the *MC3R* [8–11]. Only a single potential mutation was identified in the *MC3R* from two patients (father and daughter) in Singapore [17]. We and others showed that the mutant receptor I183N was defective in signaling [18–20]. We showed that whereas the mutant receptor bound to the ligand normally, it could not convey the ligand binding to increased Gs activation as measured by increased intracellular cAMP level. In co-transfection experiments, the mutant receptor did not affect WT MC3R binding or signaling [18]. Therefore these results would suggest that I183N might cause obesity due to haploinsufficiency rather than dominant negative activity, similar to the MC4R [32]. Lee and colleagues recently confirmed these results [20]. However, a study by Rached and coworkers showed that I183N MC3R was retained intracellularly and had dominant negative activity on WT MC3R [19]. Regardless of the exact cause of loss-of-function, all three studies showed that I183N resulted in loss of signaling in response to NDP-MSH stimulation, consistent with the hypothesis that the mutation might be the cause of increased adiposity in the patients. Recently, Feng et al. showed that the polymorphic variant T6K/V81I is associated with obesity in African Americans [31]. Very recently, two other mutations were identified from obese children [20]. Together with the results presented here for I335S, these studies suggested that *MC3R* mutations might be relevant in human adiposity. Further studies using genetically targeted animals that express the mutant MC3Rs, preferably in mouse strains that are resistant to obesity (such as Black Swiss;129) [7], are needed to confirm these in vitro studies.

With the MC4R, some of the naturally occurring mutations identified from obese patients do not cause defective signaling in vitro [22,25,33–35]. Whether these mutations were responsible for the obesity observed is not known. Similarly, in the present study, we showed that the two variants A293T and X361S did not exhibit major deleterious effect. It should be pointed out that the present experiments were done in cultured cells. Minor defect may have escaped detection by the methods used here. In addition, these variants might have defects in other aspects of receptor function such as desensitization and internalization or other signaling pathways.

Previously we suggested a classification scheme to catalogue the naturally occurring mutations in the MC4R gene associated with obesity [22]. In this scheme, Class I mutants are not expressed; Class II mutants are retained intracellularly; Class III mutants are expressed on the cell surface but defective in ligand binding; Class IV mutants can bind to the ligands but are defective in signaling; Class V mutations have no known defects [22]. According to this scheme, I335S belongs to Class II, whereas A293T and X361S belong to Class V.

The dramatic effect of I335S variant, together with the fact that I335 resides in the signature motif N/DPxxY (in the MC3R, it is DPLIY), prompted us to investigate further the role of I335 in MC3R function. Previous structure–function studies concentrated on the highly conserved Asn, Pro and Tyr (see [36–38] for examples). The two residues that are not conserved were not studied in detail. This isoleucine I335 is fully conserved in all the melanocortin receptors cloned so far (GPCR database at www.gpcr.org/7tm, version 10.0, released in June 2006). Therefore we performed additional mutagenesis studies on I335. Our studies showed that mutations of I335 into charged residues such as Asp and Arg resulted in intracellular retention, likely due to misfolding. Mutants I335A, I335L, I335N, and I335V had similar localization as the WT MC3R (Fig. 2). However, I335A and I335N did not bind to NDP-MSH, I335L exhibited minimal binding and signaling, I335V bound to NDP-MSH normally but had decreased maximal response (Fig. 4). These results highlight the importance of I335 in multiple aspects of receptor function, including cell surface expression, ligand binding, and signaling. Since I335 is located at the end of TM7, I335 might interact with hydrophobic residues in helix 8 to maintain the conformation necessary for normal trafficking, ligand binding and signaling. Valine might be able to partially interact with those residues therefore only demonstrated a decreased signaling. The other residues might result in more severe defect in forming these interactions. Further studies are needed to identify these intramolecular interactions.

Of all the inactivating mutations in GPCRs that cause human diseases, premature termination resulting in a truncated receptor is not uncommon. However, there are few examples of mutations that change the stop codon to a coding sequence resulting in a mutant receptor longer than the WT receptor. Previously a mutation in rhodopsin was found to change the stop codon to Gln resulting in 51 additional amino acids being added before the downstream stop codon [39]. The functional consequence of this mutation is not known. However, the fact that it was associated with very severe retinitis pigmentosa suggested that it might result in a significant functional defect. Another example is the GPR54, where a mutation that changes the stop codon to Arg extending the open reading frame all the way to the polyA signal. This mutation is associated with idiopathic hypogonadotropic hypogonadism [40]. The mutant receptor had decreased second messenger production [40]. We showed here that X361S MC3R did not cause major functional defects.

In summary, we provided evidences that were consistent with I335S as a possible genetic contributor for morbid obesity.

Furthermore, we showed that I335 was critical for MC3R expression, ligand binding, and signaling.

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