Endocr (2009) 36:52–59
DOI 10.1007/s12020-009-9156-4

ORIGINAL PAPER

A novel melanocortin-4 receptor gene mutation in a female patient with severe childhood obesity

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Received: 18 November 2008 / Accepted: 15 December 2008 / Published online: 12 February 2009

Abstract This study targeted the identification of mutations of melanocortin-4 receptor gene (*MC4R*) in obese children. Fifty-one unrelated probands with early onset severe obesity (body mass index (BMI) >99th percentile; 21 girls, mean age 10.6 ± 3.6 years) were analyzed for nucleotide variations in the *MC4R* coding region, by the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method followed by direct DNA sequencing. *MC4R* variants were detected in three patients: the known I169S variant was found in heterozygote state in two patients and a novel heterozygous Y302F mutation was detected in one 12-year-old girl (BMI = 34 kg/m^2, BMI *z*-score 2.7) who has been overweight since the second year of life and suffered from hyperinsulinemia (at the age of 12: fasting insulin 45 mU/ml, after oral glucose load max. 300 mU/ml). The mutation also appears in the father, although both parents are obese (BMI father: 30.2 kg/m^2; mother: 31.9 kg/m^2). This novel mutation is located in the functionally important NPXXY motif of the seventh transmembrane domain of the receptor. Functional characterization revealed reduction in cell surface expression and an alteration in signal transduction properties. These results add to the growing list of loss-of-function *MC4R* mutations in early onset obese patients and suggest an orexigenic effect of novel Y302F mutation.

Keywords Melanocortin-4 receptor gene mutation · Childhood obesity · Energy homeostasis · Decreased cell surface expression · Loss-of-function mutation

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Introduction

The control of body weight is a complex process involving interactions between genetics and environment. In early-onset obesity, the nature side of this nurture against nature paradigm weighs heavily and calls for investigations of genetic components of obesity [1]. As mutations of the melanocortin-4 receptor gene (*MC4R*) are considered the most common monogenic form of obesity [2, 3], they are the focus of this investigation. Both rodent and human studies reveal the importance of the central melanocortin pathway in controlling energy homeostasis and the pivotal role of the MC4R protein [4]. MC4R is a 332-amino acid protein of the G protein-coupled receptor (GPCR) superfamily that is highly expressed in the hypothalamic paraventricular nucleus, a region of the brain involved in appetite regulation, energy balance, and body weight control [5]. The MC4R is a seven-transmembrane GPCR and is a critical satiety signal in the leptin/melanocortin axis stimulated by α-melanocyte stimulating hormone (α-MSH)

[6]. Hypothalamic proopiomelanocortin (POMC) neurons are stimulated by leptin and represent an evident link between leptin signaling and the melanocortin system [7].

MC4R activates adenylyl cyclase via the stimulatory G protein Gs to elevate intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) levels. The endogenous ligands for the MC4R include the agonists α- and β-MSH (derived from the posttranslational processing of POMC) and the antagonist/inverse agonist agouti-related protein (AgRP). Pharmacological and mouse genetic studies establish the MC4R as one of the critical factors involved in regulating energy homeostasis [8–11]. Disruption of the MC4R signaling pathway by deletion of *MC4R* or *POMC* or overexpression of AgRP leads to hyperphagia and obesity. In humans, frameshift mutations of the *MC4R* were first reported to be associated with early onset severe obesity [12, 13]. The importance of MC4R in the regulation of body weight has been highlighted in these reports in single families, where heterozygous frameshift or nonsense mutations causing truncated proteins seemingly cosegregate with dominantly inherited early-onset obesity. Detailed functional studies of the variant MC4Rs reveal that intracellular retention of the mutant receptor is the predominant cause of its loss-of-function [14–18].

In this study we investigated *MC4R* variations in a small study group of obese children in Germany, all of whom can be described as suffering from early onset severe obesity with a mean onset age of 4.75 years, resulting in the characterization of a novel *MC4R* mutation.

Subjects and methods

Fifty-one unrelated obese children and adolescents (21 girls) from Germany (body mass index (BMI) in all patients >99th percentile, mean age 10.6 ± 3.6 years; range 3.3–17.4 years) who were consecutively treated for obesity in 2003 at the Department of Pediatrics, University of Bonn, Germany, were investigated for mutations in the *MC4R* coding region as a part of their metabolic characterization. Obesity was defined according to the International Task Force of Obesity using population-specific data [19, 20]. The mean BMI was 31.1 ± 5.18, their BMI *z*-score was 2.91 ± 0.30, and the average age at obesity onset was 4.75 ± 2.4 years. Obesity onset was defined when BMI, retrospectively evaluated, exceeded the 97th percentile for sex and age according to the reference values [20]. As BMI is not normally distributed in childhood, we used the least mean square method to calculate SDS-BMI as a measurement for the degree of overweight [21]. Written and informed consent was obtained from both parents. The study was approved by the local ethics committee of the University of Bonn, Germany, and was conducted in accordance with the guidelines of The Declaration of Helsinki. Procedures were conducted by qualified scientists adhering to the rules of respect for the individual, every patient was assured the best proven diagnostic and therapeutic method, and no one was studied without their free consent.

Anthropometrical markers and blood samples were obtained from the study group population described above. Insulin was measured by immunoassay (Immulite 2000 Analyzer; Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden), with the lowest detection at 2.0 mU/l. Blood glucose was determined by a colorimetric test (Vitros GLU-Analyseplättchen, Ortho-Clinical Diagnostics, Rochester, NY). Intra- and interassay coefficients of variation were <5%. Homeostasis model assessment (HOMA) was calculated by the following formula: resistance (HOMA-IR) = (insulin [mU/l] × glucose [mmol/l])/22.5. The median HOMA-IR of the study group was 3.3 (interquartile range 2.2–4.5). Genomic DNA was collected from nucleated white blood cells. Amplification of the *MC4R* coding region was performed using four primer pairs to generate four individual overlapping segments of 254, 260, 449, and 448 bp, Hot Taq enzyme, and an annealing temperature of 55°C for each reaction. Polymerase chain reaction (PCR) products were screened using the single-strand conformation polymorphism (SSCP) analysis (see the following section) after cutting the two larger fragments by restriction enzymes (DdeI for 449 bp and *Taq*I for 448 bp, both Roche, Germany). Subsequently, *MC4R* amplification was repeated using the same primer pairs in a buffer containing 20 mM *N*2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) titrated to pH 7.5 with Tris) (Tris–HEPES), 10 mM KCl, 10 mM ammonium sulfate, 2 mM $MgCl_2$, and the Amplitaq Gold DNA polymerase (Perkin Elmer, USA).

SSCP analysis and sequencing

In all patients, genomic DNA was isolated from peripheral blood leukocytes by standard procedures [22]. Following PCR amplification, each fragment was screened for variations employing PCR-SSCP as previously described [23, 24]. Briefly, PCR products were heat-denatured and electrophoresed on non-denaturing polyacrylamide gels. PCR products that showed aberrant migration compared to normal controls were further analyzed by direct sequencing using the Amersham-Pharmacia ALF Express automated sequencer (Pharmacia, Freiburg). Bi-directional sequencing of PCR products of all three individuals showing an aberrant SSCP pattern was performed with an automatic sequencer (ABI PRISM 310, Perkin Elmer, USA).

In vitro mutagenesis of MC4R variants

Wild-type (WT) MC4R with a myc epitope tag at the N terminus (after the initiating Met) has been described previously [17]. Mutant *MC4R*s were generated using this construct by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as detailed previously [17, 25]. Briefly, primers incorporating the desired mutations were synthesized (Invitrogen, Carlsbad, CA) and used to replace the WT sequence by PCR using myc-*MC4R* in pBlueScript as the template and *pfu* Turbo DNA polymerase (the sequences of the primers are available on request from the corresponding author). The PCR cycles consisted of denaturation at 95°C for 30 s and then 12 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 12 min. *Dpn*I was used to digest the parental methylated DNA in pBlueScript in the reaction product (37°C for 1 h), and the digested reaction product was transformed into the supercompetent XL1-Blue *Escherichia coli* cells (which repaired the nicked mutated plasmids). Individual colonies were grown and sequenced by automated DNA sequencing (performed by the DNA Core Facility at the University of Chicago Cancer Center). The plasmids with the correct mutation and without any spurious mutation introduced during PCR were ligated back into pcDNA3.1. The final constructs in pcDNA3.1 were prepared with a Maxiprep kit (Qiagen, Valencia, CA), and were sequenced again prior to transfections.

Cells and transfections

Human embryonic kidney (HEK) 293 cells 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 the gelatin-coated 35-mm six-well clusters (Corning, Corning, NY). Calcium precipitation was used to transfect HEK293 cells, with 4 μg plasmid added to each 35-mm dish [26]. In co-transfection experiments, equal amounts (2 μg each) of the WT and mutant *MC4R*s were added. Empty vector pcDNA3.1 was added to the singly transfected cells so the total amount of DNA added to each well was 4 μg. Cells were used 48 h after transfection to measure ligand binding and hormone stimulation of cAMP generation. Nonclonal cells stably expressing MC4R were established by selecting cells in growth media containing 200 μg/ml geneticin for 2 weeks after transfection. The cells were thereafter maintained in media containing geneticin. These cells were used for flow cytometry experiments.

Investigations of cell surface expression

In order to measure the levels of cell surface expression, flow cytometry of HEK293 cells stably expressing WT or mutant MC4Rs [27] were used. Cells were incubated for 1 h with fluorescein-labeled anti-myc monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 to detect the myc epitope at the N terminus of the MC4Rs and assayed with a MoFlo7-color flow cytometer & high-performance sorter (Dakocytomation, Fort Collins, CO). The expression level of the mutant was calculated as a percentage of WT expression using the formula: [mutant − pcDNA3]/[WT − pcDNA3] × 100%.

[Nle^4, D-Phe^7]-α-MSH (NDP-MSH) binding to intact cells

The binding assay has been described in detail previously [17]. Briefly, 48 h after transfection, cells were washed twice with warm Waymouth's MB752/1 media modified to contain 1 mg/ml bovine serum albumin (BSA) and 50 μg/ml gentamicin (hereafter referred to as Waymouth/BSA). Fresh Waymouth/BSA was added to each well, together with 100,000 cpm of [^{125}I]NDP-MSH (Peptide Radioiodination Service Center, The University of Mississippi, University, MS) in 50 μl, with or without different concentrations of unlabeled NDP-MSH or α-MSH (Phoenix Pharmaceuticals, Belmont, CA), and incubated at 37°C for 1 h. Then cells were placed on ice and washed twice with cold Hank's balanced salt solution containing 1 mg/ml BSA. The cells were then dissolved in 100 μl 0.5 N NaOH, collected using cotton swabs, and counted in a γ-counter. The concentrations that caused 50% inhibition (IC_{50}) were calculated using Prism 4.0 (GraphPad, San Diego, CA) software.

Signaling properties of the mutant MC4Rs

Signaling properties of the MC4Rs were assessed by measuring intracellular cAMP levels in response to NDP-MSH or α-MSH stimulation [17]. Forty-eight hours after transfection, cells were washed twice with warm Waymouth/BSA, and 1 ml of fresh Waymouth/BSA containing 0.5 mM isobutyl methylxanthine was added to each well. After 15 min incubation, either buffer alone or different concentrations of NDP-MSH or α-MSH were added, and the cells were incubated for an additional 1 h at 37°C. Intracellular cAMP was extracted with 0.5 N perchloric acid containing 180 μg/ml theophylline, and was measured by radioimmunoassay (RIA). All determinations were done in triplicate. EC_{50} values and maximal responses were calculated using Prism 4.0.

Statistical analyses

Statistical calculations were performed with SAS Edition 9.1 (one sample *t*-test) or Microsoft Excel 2003 (unpaired *t*-test). For comparisons on maximal signaling, one sample

t-test was used. For comparisons of median effective concentrations inducing 50% effect (EC_{50}) and IC_{50}, an unpaired *t*-test was used.

Results

In this study, we screened for *MC4R* variants in 51 study group members. We found a known heterozygous I169S variant in two patients and a novel Y302F mutation in one 12-year-old female patient. The novel Y302F mutation was detected by SSCP and subsequent sequencing (Fig. 1) in this female patient with early-onset obesity at the second year of life. This severely obese 12-year-old girl (BMI = 31.6 kg/m^2, >99.5th percentile, BMI *z*-score 2.7; height 160.5 cm, 75th percentile) showed acanthosis nigricans on her neck and had elevated blood pressure (150/100) measured at several time points. At the age of 12, she had marked hyperinsulinemia (insulin basal 45 mU/l, after oral glucose load max. 300 mU/l), elevated HOMA-IR (8.66), but normal glucose (fasting 78 mg/dl, max. 125 mg/dl 30 min after oral glucose load). She was heterozygous for the Y302F mutation (A905T exchange) as the mutation was also found in her father and therefore assumed to be inherited from the father (BMI father: 30.2 kg/m^2, height: 182 cm). His onset of obesity was at 10 years and he later developed hyperuricemia and high blood pressure. Her mother did not show an *MC4R* variant although she was obese (BMI mother 31.9 kg/m^2, height: 166 cm). She did not have any ancillary complications and her onset of obesity was 10 years. There were no other children in this Caucasian female patient's family. The paternal grandmother had elevated blood pressure and type 2 diabetes mellitus, and maternal and paternal grandparents were not overweight.

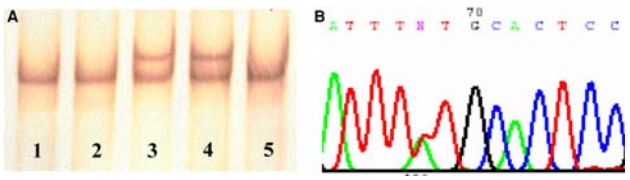

Fig. 1 Identification of the novel *MC4R* mutation Y302F. SSCP gel analysis shows abnormal pattern in lane 3 (obese girl with MC4R mutation) and lane 4 (her father). Lanes 1, 2 show unaffected controls and lane 5 her mother (**a**). Heterozygous A > T exchange in position 905 (**b**)

As mentioned above, functional studies of human MC4R polymorphisms show that I169S has normal functions compared with WT MC4R [28]. In characterizing the functional impact of the novel Y302F mutation, we reasoned that if a loss-of-function were found, it would imply that the variant was pathogenic for her early-onset obesity. Our in vitro characterization of the mutant MC4R showed that the IC_{50} in ligand binding experiments was similar to those for the WT MC4R (Table 1, Fig. 2). Using both NDP-MSH and α-MSH as ligands, we showed that the maximal signaling of the mutant was similar to the WT MC4R (Table 1, Fig. 2). The EC_{50} was increased for NDP-MSH by 6.8- and 2.4-fold for α-MSH. In co-transfection experiments, no dominant negative activity was detected when the superpotent analogue NDP-MSH was used as the ligand (Fig. 2). However, when the endogenous α-MSH was used as the ligand, a significant decrease in maximal signaling was observed, suggesting a dominant negative effect (Fig. 2). In flow cytometry experiments, the mutant had 53% of WT receptor expression, indicating decreased cell surface expression of the mutant receptor by half (Fig. 3a) and classifying this novel *MC4R* variant as a Class II mutation [29]. No difference in the constitutive activity between the WT receptor and the mutant variant was observed in the cell system and no dominant negative activity was detected in basal activity (Fig. 3b).

Discussion

As a part of the metabolic characterization of severely obese pediatric patients at the University of Bonn Pediatric Department in Bonn, Germany, we screened the entire *MC4R* coding region in 51 severely obese children and were able to identify a novel missense mutation (Y302F) as well as a known variant (I169S). The current trend indicated by several recent studies is to test any identified *MC4R* mutation for loss-of-function, although loss-of-function mutations in the *MC4R* do not necessarily lead to

Table 1 Ligand binding and agonist-stimulated cAMP generation of WT and Y302F MC4Rs

	n	NDP-MSH binding	NDP-MSH-stimulated cAMP		α-MSH binding	α-MSH-stimulated cAMP	
		IC_{50} (nM)	EC_{50} (nM)	E_{max} (% WT)	IC_{50} (nM)	EC_{50} (nM)	E_{max} (% WT)
WT	3	5.64 ± 0.54	0.85 ± 0.09	100	33.45 ± 6.00	2.46 ± 0.96	100
Y302F	3	5.17 ± 0.78	5.73 ± 1.35[a]	119 ± 3[a]	53.82 ± 11.00	5.91 ± 1.55	87 ± 11
WT/Y302F	3	4.96 ± 0.10	0.94 ± 0.15	99 ± 4	24.13 ± 2.72	1.23 ± 0.11	67 ± 6[a]

[a] Significantly different from WT receptor, $P < 0.05$

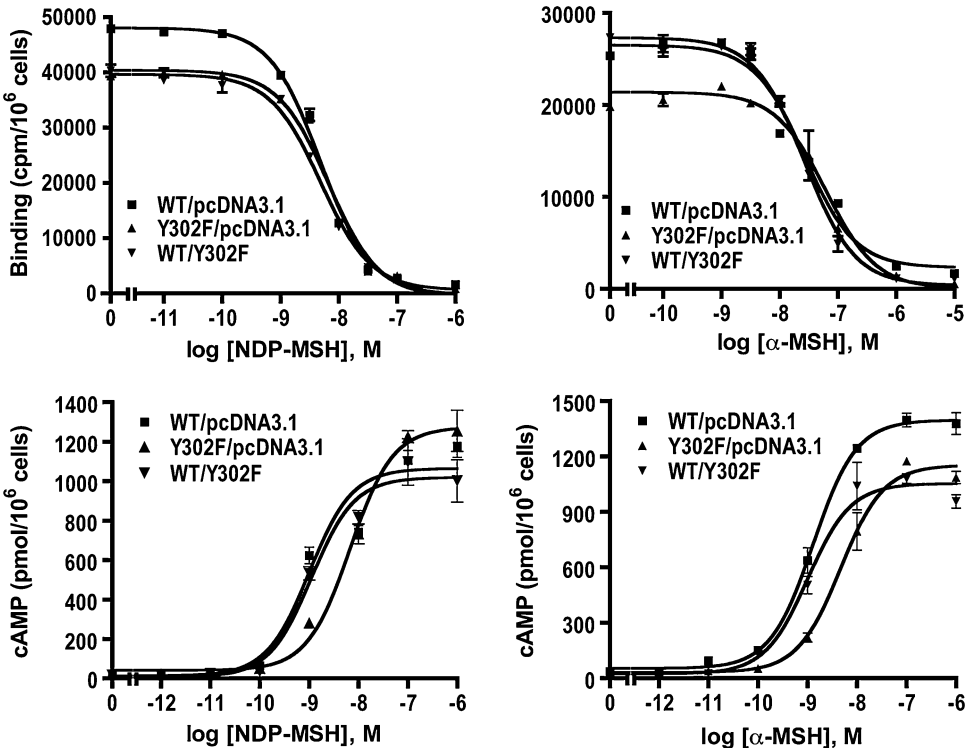

Fig. 2 Ligand binding and signaling properties of Y302F MC4R. HEK293 cells were transiently transfected with WT and/or Y302F MC4Rs and binding assays were performed as described in section "Subjects and methods." Different concentrations of unlabeled NDP-MSH (*upper left*) or α-MSH (*upper right*) were used to displace the binding of [^{125}I]NDP-MSH to WT or mutant MC4Rs on intact cells. Results shown are expressed as the mean ± range of duplicate determinations within one experiment (*upper panels*). Different concentrations of NDP-MSH (*lower left*) or α-MSH (*lower right*) were used to stimulate cells expressing WT or mutant MC4Rs. Cyclic AMP levels were measured by RIA. Results shown are expressed as mean ± SEM of triplicate determinations within one experiment (*lower panels*). All experiments were performed thrice and similar results were obtained

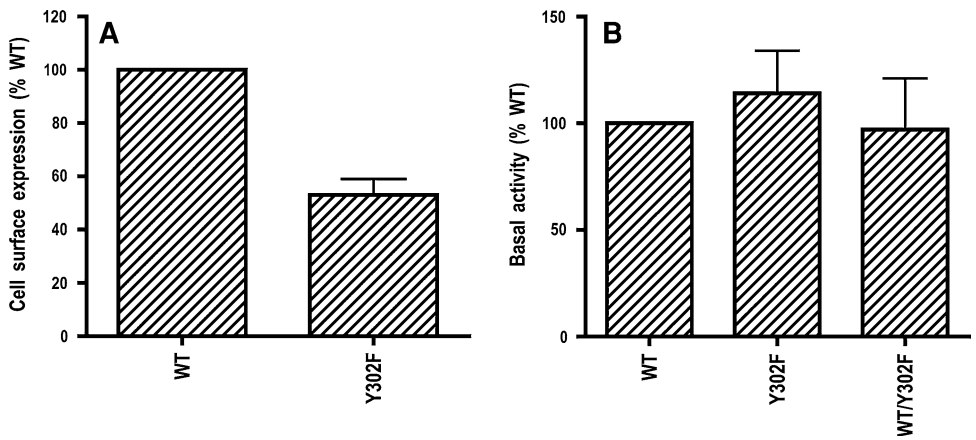

Fig. 3 Cell surface expression and constitutive activities of Y302F MC4R. Cell surface expression of MC4Rs in HEK293 cells stably expressing WT or mutant MC4Rs were measured by flow cytometry. The mutant Y302F MC4R had 53% of WT receptor expression on the cell surface (**a**). **b** The basal activities were assessed by measuring cAMP levels in cells expressing the MC4Rs in the absence of any ligand. The results are expressed as percentage of WT basal cAMP level. Shown are mean ± SEM of three or more experiments. The basal cAMP level in WT MC4R was 24.24 ± 2.56 pmol/10^6 cells (mean ± SEM of seven experiments)

obesity as the penetrance of *MC4R* mutations in causing obesity is not 100% [30].

Nevertheless, it has been established that MSHs, signaling through Gs protein/adenylyl cyclase, activate MC4R, a critical component of the hypothalamic melanocortin system involved in energy homeostasis, leading investigators to deduce that mutations in *MC4R* could be responsible for monogenic obesity in humans [12, 13] and

mice [8, 31]. MC4R loss-of-function can be characterized as decreased cell surface expression, decreased ligand binding, and/or disturbed receptor signaling. As mentioned earlier, even though loss-of-function mutations in the *MC4R* do not necessarily cause obesity, functionally relevant mutations have been reported in numerous cases of extreme obesity [32]. Furthermore, the functional impairment(s) characterizing the *MC4R* mutation result in a distinct obesity syndrome and a complete loss-of-function is associated with a more severe phenotype [33]. Interestingly, when the quantitative effect of *MC4R* mutant alleles on BMI was investigated in a family-based study design, results revealed that the allelic effect was nearly twice as strong in females [34].

It bears mentioning that not all early-onset obese patients display loss-of-function mutations. Even in our small study group, only 3 of the 51 severely obese patients we investigated revealed *MC4R* mutations and 2 of them displayed the previously reported I169S variant which does not lead to disturbed receptor signaling, decreased cell surface expression, nor decreased ligand binding [28].

In contrast to the known variant (I169S) having no loss-of-function as described above, the novel heterozygous Y302F mutation we found in one 12-year-old girl and her father revealed a 47% reduced cell surface expression (53% compared to WT receptor expression). In signaling experiments, the EC_{50} for both NDP-MSH and α-MSH was increased (Fig. 2), suggesting that the mutant is less sensitive to the agonists. As one of a family of seven transmembrane GPCRs activating adenylyl cyclase, MC4R is functionally involved in the hypothalamic regulation of food intake, implying that any loss-of-function mutation would disturb the delicate mechanism regulating homeostasis [35], an implication confirmed by a plethora of studies, several of which are cited in this report [3, 4, 8–12, 14–18, 29–34, 36–38]. It seems that the reported novel mutation (Y302F) is not a known or common MC4R variant as it was not found among 2,257 obese individuals and 2,677 nonobese control subjects of European origin as published by Stutzmann et al. [39] recently.

Amino acid Y302 lies in the highly conserved NPXXY motif in family A GPCRs (it is DPLIY in human MC4R). Although initially shown to be important for internalization in β_2-adrenergic receptor [40], later studies in other receptors showed that it is not important for internalization. Rather, it is important for receptor-G protein interaction and signaling. The receptors investigated include type 1 angiotensin II receptor [41, 42], α_{1B}-adrenergic receptor [43], gonadotropin-releasing hormone receptor [44], and B2 bradykinin receptor [45]. Saturation mutagenesis was done in serotonin 5HT2C receptor at the tyrosine residue [46]. The data obtained suggested that the tyrosine affects the isomerization of 5HT2C receptor among the myriad active and inactive conformations. Mutations of the tyrosine residue in some receptors result in no ligand binding [47, 48], whereas in some other receptors, such as gastrin-releasing peptide receptor, mutation of the conserved tyrosine to alanine does not result in any changes in ligand binding, G protein coupling or internalization [49]. In human MC4R, we showed that mutation of Y302 to alanine resulted in a defect in signaling [50]. The mutant receptor Y302A had increased affinity for NDP-MSH, but diminished signaling in response to NDP-MSH stimulation with increased EC_{50}s. The basal signaling is also decreased [50]. Therefore this mutant is in a "locked-off" state. In this study, we showed that Y302F had reduced cell surface expression but retained signaling, suggesting that the defect in Y302F is less severe than that in Y302A. In the gonadotropin-releasing hormone receptor [44], mutation of the conserved tyrosine to alanine results in loss of signaling, but mutation into phenylalanine results in a WT phenotype. Together, these results suggest that the conserved tyrosine is important for GPCR functions. The exact functions depends on the receptor.

Based on these findings it is reasonable to suppose that the phenylalanine substitution we identified results in a mutant receptor that is defective in exiting from endoplasmic reticulum. In co-transfection experiments, we showed that the mutant decreases the response of the WT receptor to the stimulation of the endogenous ligand, α-MSH, suggesting that it exerts a dominant negative effect on WT MC4R. Previously, most studies failed to show any dominant negative effect in mutant MC4Rs, even for the mutants that are retained intracellularly [29]. These findings are in sharp contrast to studies in other GPCRs that demonstrate mutant GPCRs retained intracellularly are found to retain the WT receptor in the endoplasmic reticulum by heterodimerization, therefore exerting a dominant negative effect [51]. For the MC4R, only two clear examples of dominant negative effect were found [36, 52]. We suggest that one reason might be the use of the superpotent analog NDP-MSH in many of these studies. In this study, no dominant negative activity was observed when NDP-MSH was used as the ligand. We suggest that in our female patient with the novel Y302F mutation, the obese phenotype may be a result of a mutant receptor partially retained intracellularly that has a dominant negative effect on the WT MC4R. We emphasize that regulation of homeostasis is an extremely complex system involving several organs of the body with functions often duplicated throughout these various domains.

As a correlation has been observed between the severity of the defect and of obesity [37], investigating the functional ramifications of the variants is of clinical importance. In this study, we identified a novel mutation that leads to decreased cell surface expression in a girl with

early-onset obesity that was also presented in her obese father. We are aware that this study does have some limitations. The number of examined patients is not high and potentially we did not detect all gene variants using the SSCP as the metabolic screening method we used at the time we screened this study group. But it was not our aim to calculate the prevalence of all *MC4R* variants in this area of Germany, as this had already been done by other investigators [18, 32, 34, 53]. Nevertheless, we think the functional data regarding this novel mutation contributes to an understanding of how different variants of this gene affect the receptor cell surface expression and consequently lead to a dominant negative effect.

In summary, the novel Y302F *MC4R* mutation identified in this severely obese girl with early-onset obesity and pubertal-onset diabetes is an example for dysfunctional MC4R functioning due to markedly decreased receptor expression on the cell surface. As some variants identified from obese patients have normal receptor functions, we conclude that it is important to perform in vitro functional studies of identified *MC4R* variants to decide if *MC4R* variants have a functional impact. It is also helpful to gather as much ancestral and sibling data as possible to identify hereditary patterns.

Acknowledgments The authors thank O. Hiort for his help in establishing the SSCP, and K. Harz, R. Maslak, and S. Brezinski for their support in collecting and analyzing the data. This study was supported by the Bonfor Research Foundation, Bonn, Bonfor: O-119.0010, Germany, and the Deutsche Forschungsgemeinschaft SFB 577, TP A9, NGFN-2 01GS0492TP13 and NIH grant R15DK077213.

References

1. G.S. Barsh, I.S. Farooqi, S. O'Rahilly, Genetics of body-weight regulation. Nature **404**, 644–651 (2000)
2. I.S. Farooqi, G.S.H. Yeo, J.M. Keogh, S. Aminian, S.A. Jebb, G. Butler, T. Cheetham, S. O'Rahilly, Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. J. Clin. Invest. **106**, 271–279 (2000)
3. C. Vaisse, K. Clement, E. Durand, S. Hercberg, B. Guy-Grand, P. Froguel, Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. J. Clin. Invest. **106**, 253–262 (2000)
4. A. Kask, L. Rago, J.E.S. Wikberg, H.M. Schioth, Evidence for involvement of the melanocortin MC4 receptor in the effects of leptin on food intake and body weight. Eur. J. Pharmacol. **360**, 15–19 (1998)
5. I. Gantz, H. Miwa, Y. Konda, Y. Shimoto, T. Tashiro, S.J. Watson, V. Del, J. Alle, T. Yamada, Molecular cloning, expression, and gene localisation of a fourth melanocortin receptor. J. Biol. Chem. **268**, 15174–15179 (1993)
6. R.D. Cone, Anatomy and regulation of the central melanocortin system. Nat. Neurosci. **8**, 571–578 (2005)
7. M.W. Schwartz, R.J. Seeley, S.C. Woods, D.S. Weigle, L.A. Campfield, P. Burn, D.G. Baskin, Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. Diabetes **46**, 2119–2123 (1997)
8. D. Huszar, C.A. Lynch, V. Fairchild-Huntress, J.H. Dunmore, Q. Fang, L.R. Berkemeier, W. Gu, R.A. Kesterson, B.A. Boston, R.D. Cone, F.J. Smith, L.A. Campfield, P. Burn, F. Lee, Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell **88**, 131–141 (1997)
9. L. Yaswen, N. Diehl, M.B. Brennan, U. Hochgeschwender, Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. Nat. Med. **5**, 1066–1070 (1999)
10. M.M. Ollmann, B.D. Wilson, Y.K. Yang, J.A. Kerns, Y. Chen, I. Ganty, G.S. Barsh, Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science **278**, 135–138 (1997)
11. J. Graham, J.R. Shutter, U. Sarmiento, I. Sarosi, K.L. Stark, Overexpression of Agrt leads to obesity in transgenic mice. Nat. Genet. **17**, 273–274 (1997)
12. C. Vaisse, K. Clement, B. Guy-Grand, P. Froguel, A frameshift mutation in human MC4R is associated with a dominant form of obesity. Nat. Genet. **20**, 113–114 (1998)
13. G.S.H. Yeo, I.S. Farooqi, S. Aminian, D.J. Halsall, R.G. Stanhope, S. O'Rahilly, A frameshift mutation in MC4R associated with dominantly inherited human obesity. Nat. Genet. **20**, 111–112 (1998)
14. C. Lubrano-Berthelier, E. Durand, B. Dubern, A. Shapiro, P. Dazin, J. Weill, C. Ferron, P. Froguel, C. Vaisse, Intracellular retention is a common characteristic of childhood obesity-associated MC4R mutations. Hum. Mol. Genet. **12**, 145–153 (2003)
15. G.S. Yeo, E.J. Lank, I.S. Farooqi, J. Keogh, G.B. Challis, S. O'Rahilly, Mutations in the human melanocortin4 receptor gene associated with severe familial obesity disrupts receptor function through multiple molecular mechanisms. Hum. Mol. Genet. **12**, 561–574 (2003)
16. W.A. Nijenhuis, K. Garner, R.J. Van Rozen, R.A. Adan, Poor cell surface expression of human melanocortin-4 receptor mutations associated with obesity. J. Biol. Chem. **278**, 22939–22945 (2003)
17. Y.X. Tao, D.L. Segaloff, Functional characterization of melanocortin-4 receptor mutations associated with childhood obesity. Endocrinology **144**, 4544–4551 (2003)
18. A. Hinney, A. Schmidt, K. Nottebom, O. Heibult, I. Becker, A. Ziegler, G. Gerber, M. Sina, T. Gorg, H. Mayer, W. Siegfried, M. Fichter, H. Remshmidt, J. Hebebrand, Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans. J. Clin. Endocrinol. Metab. **84**, 1483–1486 (1999)
19. M.F. Rolland-Cachera, T.J. Cole, M. Sempé, J. Tichet, C. Rossignol, A. Charraud, Variation of the Wt/Hr^2 index from birth to 87 y. Eur. J. Clin. Nutr. **45**, 13–21 (1991)
20. K. Kromeyer-Hauschild, M. Wabitsch, F. Geller, A. Ziegler, H.C. Geiss, V. Hesse, A. von Hippel, U. Jaeger, D. Johnsen, W. Kiess, W. Korte, D. Kunze, K. Menner, M. Müller, A. Niemann-Pilatus, T. Remer, F. Schaefer, H.U. Wittchen, S. Zabransky, K. Zellner, J. Hebebrand, Percentiles of body mass index in children and adolescents evaluated from different regional German studies. Monatsschr Kinderheilkd **149**, 807–818 (2001)
21. T.J. Cole, The LMS method for constructing normalized growth standards. Eur. J. Clin. Nutr. **44**, 45–60 (1990)
22. L.M. Kunkel, K.D. Smith, S.H. Boyer, D.S. Borgaonkar, S.S. Wachtel, O.J. Miller, W.R. Breg, H.W. Jones Jr, J.M. Rary, Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants. Proc. Natl. Acad. Sci. U.S.A. **74**, 1245–1249 (1977)
23. O. Hiort, Q. Huang, G.H.G. Sinnecker, A. Sadeghi-Nejad, K. Kruse, H.J. Wolfe, D.W. Yandell, Single strand conformation polymorphism analysis of androgen receptor gene mutations in patients with androgen insensitivity syndromes: application for diagnosis genetic counseling and therapy. J. Clin. Endocrinol. Metab. **77**, 262–266 (1993)

24. The German Collaborative Intersex Study Group, O. Hiort, A. Wodtke, D. Struve, A. Zöllner, G.H.G. Sinnecker, Detection of point mutations in the androgen receptor gene using non isotopic single strand conformation polymorphism analysis. Hum. Mol. Genet. **3**, 1163–1166 (1994)
25. Y.X. Tao, D.L. Segaloff, Functional characterization of melanocortin-3 receptor variants identify a loss-of-function mutation involving an amino acid critical for G protein-coupled receptor activation. J. Clin. Endocrinol. Metab. **89**, 3936–3942 (2004)
26. C. Chen, H. Okayama, High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**, 2745–2752 (1987)
27. S.X. Wang, Z.C. Fan, Y.X. Tao, Functions of acidic transmembrane residues in human melanocortin-3 receptor binding and activation. Biochem. Pharmacol. **76**, 520–530 (2008)
28. Z. Xiang, S.A. Litherland, N.B. Sorensen, B. Proneth, M.S. Wood, A.M. Shaw, W.J. Millard, C. Haskell-Luevano, Pharmacological characterization of 40 human melanocortin-4 receptor polymorphisms with the endogenous proopiomelanocortin-derived agonists and the agouti-related protein (AGRP) antagonist. Biochemistry **45**, 7277–7288 (2006)
29. Y.X. Tao, Molecular mechanisms of the neural melanocortin receptor dysfunction in severe early onset obesity. Mol. Cell. Endocrinol. **239**, 1–14 (2005)
30. Y.X. Tao, D.L. Segaloff, Functional analyses of melanocortin-4 receptor mutations identified from patients with binge eating disorder and nonobese or obese subjects. J. Clin. Endocrinol. Metab. **90**, 5632–5638 (2005)
31. T.P. Meehan, K. Tabeta, X. Du, L.S. Woodward, K. Firozi, B. Beutler, M.J. Justice, Point mutations in the melanocortin-4 receptor cause variable obesity in mice. Mamm. Genome **17**, 1162–1171 (2006)
32. A. Hinney, S. Hohmann, F. Geller, C. Vogel, C. Hess, A.K. Wermter, B. Brokamp, H. Goldschmidt, W. Siegfried, H. Remschmidt, H. Schafer, T. Gudermann, J. Hebebrand, Melanocortin-4 receptor gene: case-control study and transmission disequilibrium test confirm that functionally relevant mutations are compatible with a major gene effect for extreme obesity. J. Clin. Endocrinol. Metab. **88**, 4258–4267 (2003)
33. I.S. Farooqi, J.M. Keogh, G.S.H. Yeo, E.J. Lank, T. Chettham, S. O'Rahilly, Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. N. Engl. J. Med. **348**, 1085–1095 (2003)
34. A. Dempfle, A. Hinney, M. Heinzel-Gutenbrunner, M. Raab, F. Geller, T. Gudermann, H. Schafer, J. Hebebrand, Large quantitative effect of melanocortin-4 receptor gene mutations on body mass index. J. Med. Genet. **10**, 795–800 (2004)
35. M.W. Schwartz, S.C. Woods, D. Porte Jr., R.J. Seeley, D.G. Baskin, Central nervous system control of food intake. Nature **404**, 661–671 (2000)
36. H. Biebermann, H. Krude, A. Elsner, V. Chubanov, T. Gudermann, A. Grüters, Autosomal-dominant mode of inheritance of a melanocortin-4 receptor mutation in a patient with severe early-onset obesity is due to a dominant-negative effect caused by receptor dimerization. Diabetes **52**, 2984–2988 (2003)
37. J. Grosse, P. Tarnow, H. Römpler, B. Schneider, R. Sedlmeier, U. Huffstadt, D. Korthaus, M. Nehls, S. Wattler, T. Schöneberg, H. Biebermann, M. Augustin, N-ethyl-N-nitrosourea-based generation of mouse models for mutant G protein-coupled receptors. Physiol. Genomics **26**, 209–217 (2006)
38. G. Ho, R.G. MacKenzie, Functional characterization of mutations in melanocortin-4 receptor associated with human obesity. J. Biol. Chem. **274**, 35816–35822 (1999)
39. F. Stutzmann, K. Tan, V. Vatin, C. Dina, B. Jouret, J. Tichet, B. Balkau, N. Potoczna, F. Horber, S. O'Rahilly, I.S. Farooqi, P. Froguel, D. Meyre, Prevalence of melanocortin-4 receptor deficiency in Europeans and their age-dependent penetrance in multigenerational pedigrees. Diabetes **57**, 2511–2518 (2008)
40. L.S. Barak, M. Tiberi, N.J. Freedman, M.M. Kwatra, R.J. Lefkowitz, M.G. Caron, A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated β2-adrenergic receptor sequestration. J. Biol. Chem. **269**, 2790–2795 (1994)
41. L. Hunyady, M. Bor, A.J. Baukal, T. Balla, K.J. Catt, A conserved NPLFY sequence contributes to agonist binding and signal transduction but is not an internalization signal for the type 1 angiotensin II receptor. J. Biol. Chem. **270**, 16602–16609 (1995)
42. S.A. Laporte, G. Servant, D.E. Richard, E. Escher, G. Guillemette, R. Leduc, The tyrosine within the NPXnY motif of the human angiotensin II type 1 receptor is involved in mediating signal transduction but is not essential for internalization. Mol. Pharmacol. **49**, 89–95 (1996)
43. J. Wang, J. Zheng, J.L. Anderson, M.L. Toews, A mutation in the hamster α_{1B}-adrenergic receptor that differentiates two steps in the pathway of receptor internalization. Mol. Pharmacol. **52**, 306–313 (1997)
44. K.K. Arora, Z. Cheng, K.J. Catt, Dependence of agonist activation on an aromatic moiety in the DPLIY motif of the gonadotropin-releasing hormone receptor. Mol. Endocrinol. **10**, 979–986 (1996)
45. I. Kalatskaya, S. Schussler, A. Blaukat, W. Muller-Esterl, M. Jochum, D. Proud et al., Mutation of tyrosine in the conserved NPXXY sequence leads to constitutive phosphorylation and internalization, but not signaling, of the human B2 bradykinin receptor. J. Biol. Chem. **279**, 31268–31276 (2004)
46. C. Prioleau, I. Visiers, B.J. Ebersole, H. Weinstein, S.C. Sealfon, Conserved helix 7 tyrosine acts as a multistate conformational switch in the 5HT2C receptor. Identification of a novel "locked-on" phenotype and double revertant mutations. J. Biol. Chem. **277**, 36577–36584 (2002)
47. W. Feng, Z.H. Song, Functional roles of the tyrosine within the NP(X)(n)Y motif and the cysteines in the C-terminal juxtamembrane region of the CB2 cannabinoid receptor. FEBS Lett. **501**, 166–170 (2001)
48. N. Hukovic, R. Panetta, U. Kumar, M. Rocheville, Y.C. Patel, The cytoplasmic tail of the human somatostatin receptor type 5 is crucial for interaction with adenylyl cyclase and in mediating desensitization and internalization. J. Biol. Chem. **273**, 21416–21422 (1998)
49. L.W. Slice, H.C. Wong, C. Sternini, E.F. Grady, N.W. Bunnett, J.H. Walsh, The conserved NPXnY motif present in the gastrin-releasing peptide receptor is not a general sequestration sequence. J. Biol. Chem. **269**, 21755–21761 (1994)
50. Y.X. Tao, The functions of DPLIY motif and helix 8 in human melanocortin-4 receptor. Program & Abstracts of the Endocrine Society's 88th Annual Meeting,2006, p. 243 (Abstract P1-327)
51. Y.X. Tao, Inactivating mutations of G protein-coupled receptors and diseases: structure-function insights and therapeutic implications. Pharmacol. Ther. **111**, 949–973 (2006)
52. P. Tarnow, A. Rediger, H. Brumm, P. Ambrugger, E. Rettenbacher, K. Widhalm, A. Hinney, G. Kleinau, M. Schaefer, J. Hebebrand, G. Krause, A. Grüters, H. Biebermann, A heterozygous mutation in the third transmembrane domain causes a dominant-negative effect on signaling capability of the MC4R. Obes. Facts **1**, 155–162 (2008)
53. T. Reinehr, A. Hinney, G. de Sousa, F. Austrup, J. Hebebrand, W. Andler, Definable somatic disorders in overweight children and adolescents. J. Pediatr. **150**, 618–622 (2007)