Brief Genetics Report

Co-occurrence of Two Partially Inactivating Polymorphisms of *MC3R* Is Associated With Pediatric-Onset Obesity

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Both human linkage studies and *MC3R* knockout mouse models suggest that the *MC3R* may play an important role in energy homeostasis. Here we show that among 355 overweight and nonoverweight children, 8.2% were double homozygous for a pair of missense *MC3R* sequence variants (Thr6Lys and Val81Ile). Such children were significantly heavier (BMI and BMI SD score: $P < 0.0001$), had more body fat (body fat mass and percentage fat mass: $P < 0.001$), and had greater plasma leptin ($P < 0.0001$) and insulin concentrations ($P < 0.001$) and greater insulin resistance ($P < 0.008$) than wild-type or heterozygous children. Both sequence variants were more common in African-American than Caucasian children. In vitro expression studies found the double mutant *MC3R* was partially inactive, with significantly fewer receptor binding sites, decreased signal transduction, and less protein expression. We conclude that diminished *MC3R* expression in this double *MC3R* variant may be a predisposing factor for excessive body weight gain in children. *Diabetes* 54:2663–2667, 2005

Both human linkage studies and knockout mouse models suggest that *MC3R* (the melanocortin 3 receptor gene) plays an important role in energy homeostasis (1–5). Mice in whom both alleles of the *MC3R* have been inactivated have greater fat mass and reduced lean mass, while heterozygotes have normal body composition (3). Three human coding sequence variants have been reported in the *MC3R* (6–10): a heterozygous missense sequence variant Ile183Asn (I183N) that inactivates *MC3R* function (11,12), identified in two individuals (10), and two other variants, Thr6Lys and Val81Ile, that individually do not affect signal transduction (12) and have been reported not to be associated with body weight (6–9). Because most single gene mutations affecting body weight become manifest during childhood, we performed an *MC3R* mutation screening and association analysis in a large cohort of African-American and Caucasian overweight and normal weight children and then performed functional studies for the sequence variants that were identified.

RESEARCH DESIGN AND METHODS

A total of 355 children aged 5–18 years (mean age 10.7 ± 3.1 years) were recruited from the community for metabolic studies at the National Institutes of Health. By design, the study population was enriched for obesity: 190 were overweight, with BMI ≥95th percentile for age and sex, and 165 were nonoverweight (BMI 5th to 94th percentiles). No subject had significant medical illness, and none were taking medications known to affect body weight. The National Institute of Child Health and Human Development institutional review board approved the clinical protocol. Signed consent and assent forms were obtained from parents and children.

Phenotypic data. BMI-SD score, body adiposity, and body circumferences as well as metabolic parameters were obtained as previously described (13–15). The homeostasis model assessment for insulin resistance index (HOMA-IR) (16) was estimated using the formula HOMA-IR = [fasting insulin (μU/ml) × fasting glucose (mmol/l)]/22.5.

Mutation screening and genotyping. After PCR amplification of genomic DNA, we directly sequenced the human *MC3R* (L06155), including a 336 bp of proximal promoter and the entire coding region, in 47 extremely overweight (BMI 41.8 ± 10.5 kg/m^2) and 31 lean (BMI 16.8 ± 2.2 kg/m^2) children. Restriction enzyme fragment length polymorphism analysis of identified variants was used for genotyping the entire cohort.

Construction and expression of *MC3R*. Wild type, Thr6Lys, and Val81Ile single mutants and Thr6Lys+Val81Ile double mutant *MC3R* (i.e., *MC3R* sequence altered to introduce both mutations into the same gene) were constructed in pcDNA3.1/V5-His TOPO vectors (Invitrogen, Carlsbad, CA) without expressing the V5 or His tag. Wild type and the Thr6Lys+Val81Ile double mutant were also constructed with a C-terminus enhanced green fluorescence protein (EGFP) vector, pEGFP-N2 (BD Biosciences, San Jose, CA). Constructs were transiently expressed in HEK-293 or LVIP2.0Zc cells. LVIP2.0Zc is a reporter cell line containing β-galactosidase driven by a CRE-dependent promoter (17). Transfections were performed in Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, CA) using Lipofectamine Plus reagent (Invitrogen).

Receptor binding assay. Ligand binding studies were carried out in HEK-293 cells exposed to 100,000 cpm of [^{125}I] (Nle4,D-Phe7) α-melanocyte stimulating hormone (NDP α-MSH) (Amersham, Piscataway, NJ) and increasing concentrations of cold NDP α-MSH (Peninsula, San Carlos, CA) in the range of

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DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescence protein; FACS, fluorescence-assisted cell sorting; MSH, melanocyte-stimulating hormone; NDP α-MSH, (Nle4,D-Phe7) α-melanocyte-stimulating hormone.

TABLE 1
Study subject demographics grouped by *MC3R* variant genotype

	Wt/Wt*	Het†	Hom/Hom‡	*P*
n	202	124	29	
Age (years)	10.6 ± 3.0§	10.8 ± 3.2	10.9 ± 2.9	NS
Sex				NS
Female	53.9 (110)	36.8 (75)	9.3 (19)	
Male	60.9 (92)	32.4 (49)	6.6 (10)	
Race				<0.0001
African American	31.6 (48)	52.6 (80)	15.8 (24)	
Caucasian	80.1 (141)	18.2 (32)	1.7 (3)	
Other	48.2 (13)	44.4 (12)	7.4 (2)	

Data are % (*n*), unless otherwise indicated. *Sequence variants were identified by restriction fragment length polymorphism analysis using enzymes HpyCH4 IV to identify the Thr6Lys variant and BsaJ I to identify the Val81Ile variant. Wt/Wt, wild type for both alleles (Thr/Thr and Val/Val); †Het, heterozygous for at least one allele (Thr/Thr and Val/Ile, Thr/Lys and Val/Ile, Lys/Lys and Val/Ile, or Thr/Lys and Ile/Ile); ‡Hom/Hom, homozygous for both variant alleles (Lys/Lys and Ile/Ile); §means ± SD.

10^{-10}–10^{-6} mmol/l. After 4 h of incubation at room temperature, membrane-bound counts per minute were determined in a gamma counter.

β-Galactosidase activity measurement and cAMP assay. Ligand-stimulated receptor activity was determined by β-galactosidase activity in transfected LVIP2.0Zc reporter cells. β-galactosidase activity was measured using a β-galactosidase enzyme assay system (Promega, Madison WI). Ligand-stimulated intracellular cAMP accumulation was also directly measured in transfected HEK293 cells incubated with NDP α-MSH in the presence of 1 mmol/l 3-Isobutylmethylxanthine (Sigma, St. Louis, MO).

Confocal imaging, fluorescence-assisted cell sorting (FACS) and Western analysis. EGFP-tagged wild-type and double mutant *MC3R* were transiently transfected in HEK293 cells and studied 48 h posttransfection. For confocal imaging, cells were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature and washed with PBS. Images were captured using a Zeiss 510 confocal microscope. For FACS, trypsin-treated cells were pelletted by centrifugation and then resuspended in 1× PBS to a concentration of 1×10^6 cells/ml. Sorting data were collected with a FACS calibur flow cytometer and analyzed with Cell Quest software (Becton Dickinson, San Jose, CA). For Western analysis, whole cell lysates were separated on 10–20% SDS-PAGE gels, electrotransferred onto nitrocellulose membranes, and immunoblotted with a mouse monoclonal anti–green fluorescent protein antibody (Clontech, Palo Alto, CA).

Data analysis. Comparisons of the phenotypes by genotype were evaluated by using ANCOVA, accounting for age, height, race, and sex. Because of the multiple comparisons performed, a more conservative $P < 0.0125$ was accepted as significant for ANCOVAs. GraphPad Prism 4.0 (GraphPad Software, San Diego, CA) was used for calculation of Scatchard analysis data.

RESULTS

By sequencing, we found two previously identified missense sequence variants: Thr6Lys and Val81Ile. Among the 355 children studied, the two variants showed marked linkage disequilibrium ($x^2 = 522.4$, $P < 0.0001$). Therefore, the genotypes were categorized based on combination of the two variants (Table 1). African-American children were more likely to carry the variant alleles ($P < 0.0001$ vs. Caucasians): 15.8% (vs. 1.7% of Caucasians) were homozygous for both polymorphisms. Overweight children had higher frequencies of the variants than normal weight children ($x^2 = 21.31$, $P < 0.001$). Children homozygous for both variants were identified only among children who were at risk for overweight (BMI ≥85th percentile, 7.9%) or who were considered overweight (BMI ≥95th percentile, 13.7%).

Children who were homozygous for both *MC3R* variants had significantly greater BMI-SD score, fat mass, body circumference measurements, insulin, and leptin compared with wild-type or heterozygous children (Table 2). Analysis by race showed greater BMI-SD score and dual-energy X-ray absorptiometry body fat mass in African-American children who were double-homozygous for the two *MC3R* alleles. There were only three Caucasian children who were double-homozygous for the variants; trends of associations were also found between body composition and the *MC3R* variants in Caucasians. *MC4R* was also sequenced for each child who was double ho-

TABLE 2
Subject characteristics according to *MC3R* genotype

	Wt/Wt*	Het†	Hom/Hom‡	*P*
n	202	124	29	
BMI (kg/m^2)	25.6 ± 11.4	27.5 ± 12.6	35.0 ± 12.5	<0.0001
BMI SD score	2.4 ± 3.2	2.8 ± 3.2	5.3 ± 3.4	<0.0001
Fat mass (kg)	23.9 ± 21.5	23.7 ± 20.9	40.1 ± 24.0	<0.001
Fat mass (%)	33.3 ± 13.6	33.8 ± 12.2	43.6 ± 7.4	<0.0001
Body circumferences (cm)				
Midarm	31.6 ± 10.0	33.8 ± 10.1	38.2 ± 8.2	0.004
Chest	92.5 ± 23.5	96.4 ± 22.1	108.0 ± 18.6	0.003
Waist	85.5 ± 24.4	88.1 ± 23.2	100.3 ± 20.4	0.016
Abdomen	92.1 ± 28.1	93.5 ± 26.2	110.1 ± 21.5	0.005
Hip	98.8 ± 26.2	101.4 ± 26.1	117.4 ± 24.7	<0.0001
Thigh	55.2 ± 16.0	58.5 ± 16.4	69.2 ± 16.4	<0.0001
Hormones and substrates				
Glucose (mg/dl)	87.5 ± 14.9	89.2 ± 15.4	89.6 ± 7.2	NS
Insulin (uIU/l)	13.3 ± 13.7	16.0 ± 15.2	24.7 ± 15.7	0.006
HOMA-IR	2.9 ± 3.2	3.6 ± 3.6	5.4 ± 3.6	0.008
Triglyceride (mg/dl)	81.3 ± 49.4	89.0 ± 90.4	91.5 ± 56.3	NS
Total cholesterol (mg/dl)	167.0 ± 31.5	172.5 ± 31.3	169.0 ± 28.9	NS
LDL cholesterol	107.1 ± 27.5	112.4 ± 29.0	113.2 ± 26.1	NS
Serum leptin (mg/dl)	11.4 ± 11.7	12.9 ± 13.8	26.3 ± 16.6	<0.0001

Data are means ± SD. *Wt/Wt, wild type for both alleles (Thr/Thr and Val/Val); †Het, heterozygous for at least one allele (Thr/Thr and Val/Ile, Thr/Lys and Val/Ile, Lys/Lys and Val/Ile, or Thr/Lys and Ile/Ile); ‡Hom/Hom, homozygous for both variant alleles (Lys/Lys and Ile/Ile). HOMA-IR, homeostasis model assessment for insulin resistance.

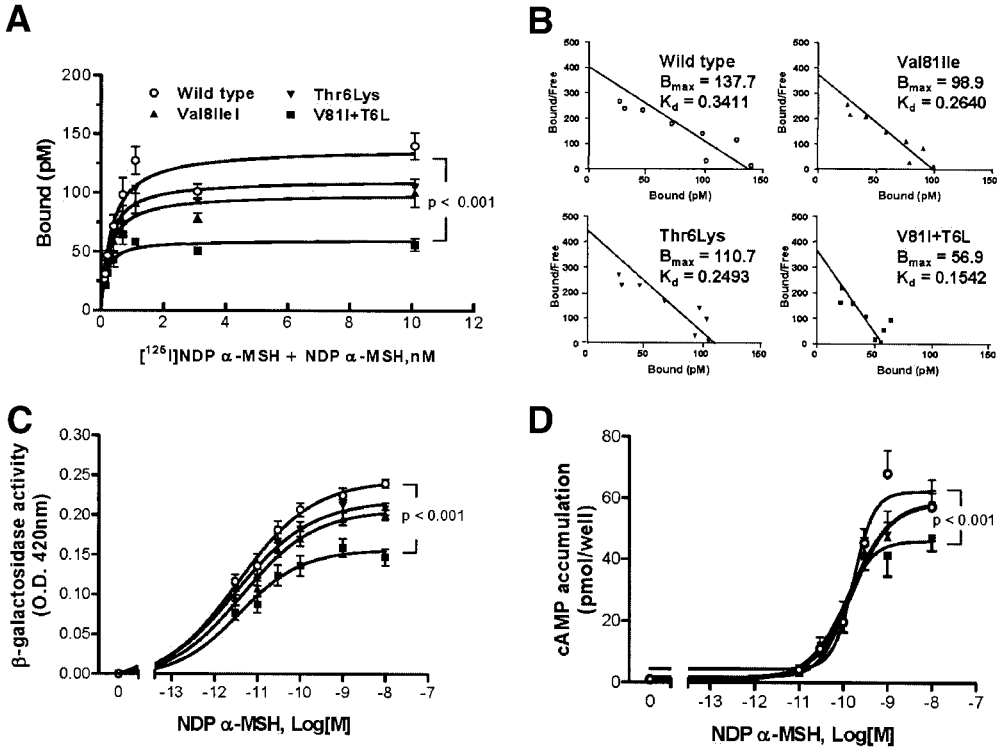

FIG. 1. In vitro studies of the *MC3R* variants. *A*: Saturation curve of NDP α-MSH binding to mutant or wild-type *MC3R* in transfected HEK-293 cells (data from four independent experiments, $n = 12$). *B*: Scatchard analysis plotted from the data shown in Fig. 1*B*. *C*: NDP α-MSH–stimulated β-galactosidase activity in transfected LVIP2.0ZC cells with the variants or wild-type *MC3R* (data from four independent experiments, $n = 12$). *D*: NDP α-MSH–stimulated intracellular cAMP generation in transfected HEK-293 cells with variant or wild-type *MC3R* (data from three independent experiments, $n = 9$). Ligand binding studies were carried out in HEK-293 cells 48 h post transfection in 24-well plates. After washing with PBS, cells were treated with 500 μl of binding buffer (Dulbecco's modified Eagle's media [DMEM] with 25 mmol/l HEPES, 100 kIU/ml of aprotinin, and 0.1% of BSA), containing ~100,000 cpm of [^{125}I] NDP α-MSH and increasing concentrations of cold NDP α-MSH in the range of 10^{-10}–10^{-6} mmol/l. After 4 h of incubation at room temperature, cells were washed with ice-cold PBS containing 0.1% BSA and then lysed in 0.4 ml of 0.1N NaOH. For β-galactosidase activity studies, transfected LVIP2.0ZC cells were cultured in 24-well plates and incubated with NDP α-MSH in DMEM with 100 kIU/ml of aprotinin and 0.1% of BSA. After 6 h, cells were washed with PBS and then lysed in 200 μl of 1× Reporter Lysis Buffer (Promega, Madison, WI). For cAMP measurements, HEK-293 cells cultured in 24-well plates were incubated with NDP α-MSH in the presence of 1 mmol/l 3-Isobutylmethylxanthine (Sigma, St. Louis, MO) in DMEM with 100 kIU/ml aprotinin and 0.1% BSA. After 30 min, cells were washed with PBS and then lysed in 400 μl of 0.1N HCL. HCL extracts were assayed for intracellular cAMP using a direct cAMP enzyme immunosorbent assay kit (Assay Designs, Ann Arbor, MI).

mozygous for these *MC3R* variants (data not shown). No function-altering mutations were found.

To study the functional consequences of these *MC3R* variants, we studied ligand binding and melanocyte-stimulating hormone (MSH)-stimulated cAMP generation of single and double mutant *MC3R* variants. By Northern analysis (data not shown), mRNA expression was similar for wild-type and mutant receptors. Saturation curves for binding of ^{125}I NDP α-MSH (Fig. 1*A*) suggested the double mutant Thr6Lys and Val81Ile *MC3R* bound ~60% less [^{125}I] NDP α-MSH than the wild-type *MC3R* ($P < 0.001$). Scatchard analysis revealed a similar binding affinity for variant *MC3R*s (Fig. 1*B*). However, the level of surface expression, as reflected by estimated B_{max}, was significantly lower for the double mutant *MC3R* (56.9 pmol/l) compared with the wild-type *MC3R* (137.7 pmol/l). Functional activity of the mutants was in accord with their cell surface expression: the Val81Ile and Thr6Lys single mutant *MC3R*s exhibited a dose-dependent pattern of response to NDP-MSH not significantly different from wild-type *MC3R*. However, the Val81Ile+Thr6Lys double mutant *MC3R*, studied using two cAMP measurement systems (Fig. 1*C* and *D*), showed significantly reduced intracellular cAMP generation. Consistent with the receptor affinity data, the half-maximal effective NDP α-MSH concentration to stimulate cAMP was similar for all constructs (Val81Ile+Thr6Lys 0.11 ± 0.15 vs. wild type 0.14 ± 0.13 nmol/l).

To study protein expression and localization of mutant *MC3R*s, we studied *MC3R* variants tagged with EGFP. Similar to the untagged receptors, cAMP generation was markedly reduced in the EGFP-tagged double mutant *MC3R* (Figs. 2*A* and *B*). By confocal microscopy, both wild-type and double mutant EGFP-tagged *MC3R*s were expressed in the plasma membrane (Fig. 2*C*). However, by FACS (Fig. 2*D*), the double mutant had significantly less total EGFP signal, suggesting a reduced receptor protein content. This was confirmed by Western blot analysis of the EGFP-tagged receptor (Fig. 2*E*).

DISCUSSION

We found that the co-occurrence of two previously identified variants in *MC3R* was associated with impaired cAMP generation in vitro and with greater BMI, greater body fat mass, and higher plasma levels of insulin and leptin in African-American and Caucasian children. To our knowledge, although others have examined the relationships with BMI (6–9) and the functional consequences (8,12) of these two polymorphisms separately, the present

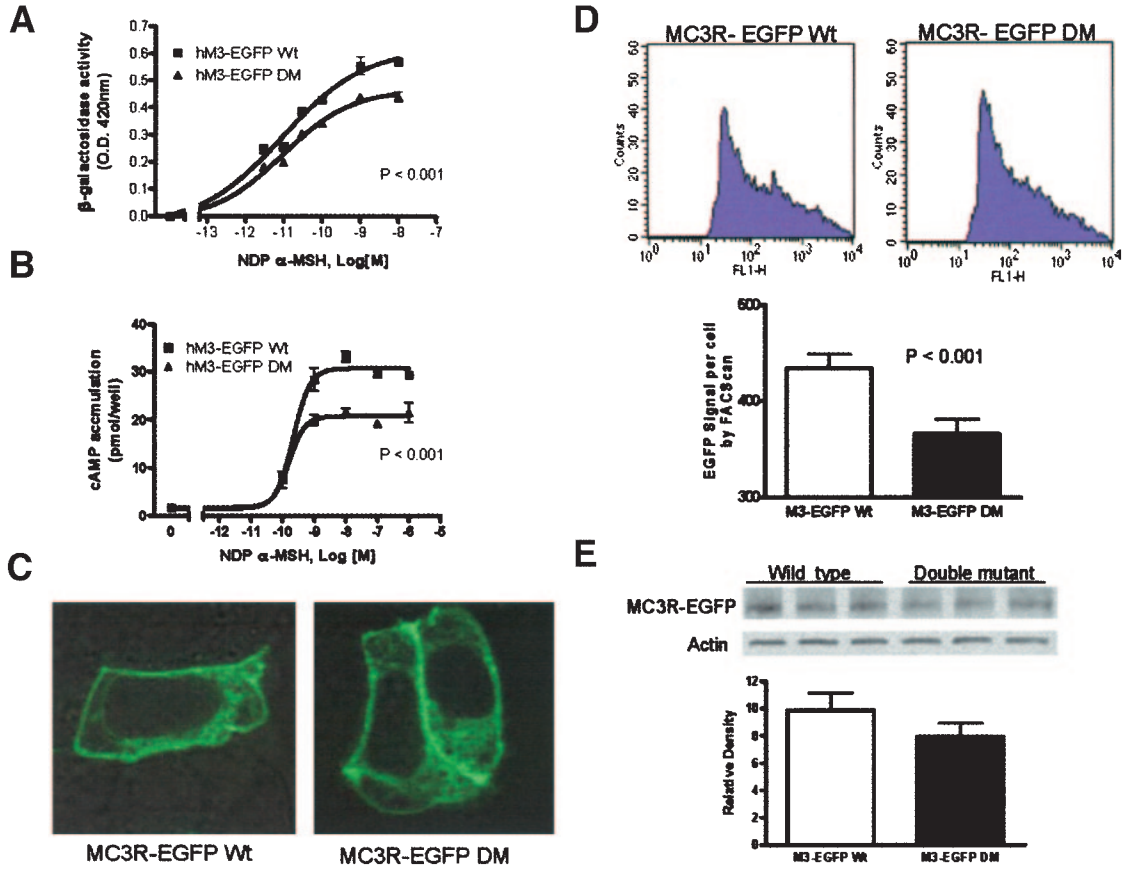

FIG. 2. In vitro studies of *MC3R* variants tagged with EGFP. *A*: NDP α-MSH–stimulated β-galactosidase activity in transfected LVIP2.0ZC cells with the Thr6Lys+Val81Ile double mutant (DM) or wild-type *MC3R* (Wt). *B*: NDP α-MSH–stimulated intracellular cAMP generation in transfected HEK-293 cells with DM or Wt. *C*: Localization of the DM and Wt *MC3R* by confocal imaging; images were captured using a Zeiss 510 confocal microscope equipped with a 488 nm laser for excitation and a LP505 emission filter and a Plan-Apochromat 63×/1.4 oil immersion objective (Carl Zeiss, Jena, Germany). *D*: Quantitation of *MC3R*-EGFP protein expression by fluorescence-assisted cell sorting. Representative sorting of *MC3R*-EGFP signal cells for Wt and DM (*top panels*) and the average fluorescent intensity from three independent experiments (*bottom panel*). *E*: Immunoblotting analysis with a mouse monoclonal anti–green fluorescent antibody (Clontech, Palo Alto, CA) at a dilution of 1:2,000. Representative blot (*top panel*) and quantitation from three independent experiments (*bottom panel*).

report is the first investigation to study body composition for those who are double homozygotes and to study signal transduction in the double variant *MC3R*. The association we found between high body weight and double homozygosity in children is consistent with findings that both *MC3R* alleles must be impaired for increased body adiposity to be observed in *MC3R* knockout animal models. The failure to observe a relationship between these variants and BMI in prior studies may be due to the fact that large clinical samples enriched with double homozygotes have not previously been examined. One study (6) of 24 obese adults and 27 control subjects reported 12% of African-American women were homozygous for these polymorphisms but failed to find an association with body weight. It is possible that insufficient numbers of African Americans were previously studied to show the impact of the presence of both *MC3R* variants or, since some nonobese adults are reported to be double homozygotes (6), that these variants are more associated with the development of overweight during childhood than adulthood. It is also theoretically possible that the strong association observed in the present study could be due to linkage disequilibrium with another nearby gene locus on chromosome 20 or could have occurred by chance.

Thr6Lys and Val81Ile are found, respectively, in the NH_2-terminal extracellular part and the first transmembrane helix of *MC3R* protein. Mutations in either location might be predicted to affect melanocortin receptor function, especially the first transmembrane region that is believed to be involved in the binding of melanocortin peptides (18). The complete binding curves in our study show that the double mutant had significantly less total ligand binding capacity in spite of preserved binding affinity, similar to findings with some *MC4R* variants reported to be associated with obesity (19). The reduced binding in our experiments was consistent with the decreased MSH-stimulated cAMP production by the double mutant receptor, shown by direct cAMP measurements and indirectly using cAMP-responsive reporter cells. The mechanism of the lower activity of the double mutant *MC3R* appears to involve less receptor protein expression, as suggested by both FACS and Western blot analysis. The lower receptor protein levels for this mutant did not depend on transfection efficiency or mRNA levels (data not shown) but appears due to posttranslational events. It remains unclear whether the lower protein expression is due to decreased synthesis or increased degradation. Although the double mutation led to lower protein expression, membrane localization of this receptor appeared to be similar to that of the wild-type receptor by confocal

microscopy; thus, intracellular retention, hypothesized to be the cause of the inactivity of some *MC4R* variants (20), appears unlikely.

The mechanism for increased adiposity among *MC3R*-deficient mice appears to be increased feeding efficiency, such that the same energy intake results in greater storage of calories as fat (11). Further studies should examine whether co-occurrence of these *MC3R* polymorphisms affects energy balance or efficiency.

Limitations of the present study include evaluation of only one cohort of children and the small number of Caucasians homozygous for these variants. The limitations of in vitro assays for receptor function should also be noted, as results potentially may differ in vivo. Finally, receptor function was evaluated only using an analog of α-MSH and not with γ-MSH, which is also a potent agonist for the *MC3R* (21). However, prior studies do not suggest marked differences in response of the *MC3R* to α- versus γ-MSH (22).

In summary, double homozygocity for *MC3R* sequence variants Thr6Lys and Val81Ile is particularly prevalent in African-American children and is associated with adiposity and insulin resistance in African-American and Caucasian children, and in vitro partially inactivates the *MC3R*. Double homozygocity for this *MC3R* mutant may contribute to the greater prevalence of overweight and insulin resistance in African Americans.

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