Heterozygous Mutations Causing Partial Prohormone Convertase 1 Deficiency Contribute to Human Obesity

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Null mutations in the *PCSK1* gene, encoding the proprotein convertase 1/3 (PC1/3), cause recessive monogenic early onset obesity. Frequent coding variants that modestly impair PC1/3 function mildly increase the risk for common obesity. The aim of this study was to determine the contribution of rare functional *PCSK1* mutations to obesity. *PCSK1* exons were sequenced in 845 nonconsanguineous extremely obese Europeans. Eight novel nonsynonymous *PCSK1* mutations were identified, all heterozygous. Seven mutations had a deleterious effect on either the maturation or the enzymatic activity of PC1/3 in cell lines. Of interest, five of these novel mutations, one of the previously described frequent variants (N221D), and the mutation found in an obese mouse model (N222D), affect residues at or near the structural calcium binding site Ca-1. The prevalence of the newly identified mutations was assessed in 6,233 obese and 6,274 lean European adults and children, which showed that carriers of any of these mutations causing partial PCSK1 deficiency had an 8.7-fold higher risk to be obese than wild-type carriers. These results provide the first evidence of an increased risk of obesity in heterozygous carriers of mutations in the *PCSK1* gene. Furthermore, mutations causing partial PCSK1 deficiency are present in 0.83% of extreme obesity phenotypes. ***Diabetes*** **61:383–390, 2012**

Proprotein convertase 1/3 (PC1/3, gene symbol *PCSK1*) represents the major processing enzyme of precursor proteins in the regulated secretory pathway and is expressed in the brain, enteroendocrine cells, and neuroendocrine system (1–3). PC1/3 is synthesized as inactive proPC1/3, which is rapidly converted into PC1/3 by autocatalytic cleavage of the NH_2-terminal propeptide in the endoplasmic reticulum (ER) (4,5). A second internal cleavage of the propeptide in a post-ER compartment is required for activation (6). COOH-terminal processing of PC1/3 occurs in a late- or post-Golgi compartment, a process that affects enzyme kinetics and stability (7). On the basis of the crystal structure of the PC furin, two calcium binding sites are predicted. The Ca-1 site is needed for structural stabilization, and the Ca-2 site is essential for the formation of the P1 specificity–determining S1-binding pocket (8–10).

Three patients with recessive monogenic forms of obesity due to total PCSK1 deficiency have been identified (6,11,12). These mutations cause early onset obesity, hyperphagia, reactive hypoglycemia, and (entero)endocrine dysfunctions. In the three studies, probands were either compound heterozygous or homozygous for mutations in *PCSK1*. The eight heterozygous family members appeared clinically unaffected and not obese.

Although *PCSK1*-null mice are not obese, they display growth retardation and multiple neuroendocrine abnormalities (13). In contrast, heterozygote *PCSK1-null* mice are not growth retarded but tend to be mildly obese. Mice homozygous for the deleterious N222D mutation are obese with abnormal proinsulin processing and multiple endocrinological defects (14). N222D-heterozygous mice are characterized by an intermediate phenotype and display an increased body fat content compared with wild-type mice.

Recently, we suggested the contribution of *PCSK1* common nonsynonymous polymorphisms (N221D and the Q665E-S690T cluster) to polygenic obesity in European populations, placing *PCSK1* on the list of genes associated with this common disease (15). Although the single nucleotide polymorphism (SNP) N221D has a modest effect on PC1/3 activity, it increased the risk for obesity. Since this initial report, associations of the N221D or the Q665E-S690T polymorphisms with obesity-related traits have been reported in at least subgroup analyses of several independent replication studies (16–21). However, frequent coding SNPs in *PCSK1* as well as all others recently identified through Genome Wide Association Studies explain only a small fraction of obesity heritability (17,19,22–24). In addition to common variants, rare variants that have stronger functional effect are therefore expected to play an important role in the genetics of common diseases (25).

We hypothesized that rare heterozygous mutations in *PCSK1* may contribute to severe forms of obesity. Therefore, we have sequenced coding regions of *PCSK1* in 845 extremely obese subjects and compared our data with the DNA sequences available from the public human genome

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databases. A combination of in silico and in vitro characterization was applied to the eight detected nonsynonymous mutations to evaluate their consequences on the maturation and activity of PC1/3. Finally, the eight mutations have been genotyped in 6,233 obese and 6,274 lean European subjects to estimate their association to obesity risk.

RESEARCH DESIGN AND METHODS

The study protocol has been approved by all local ethics committees, and informed consent was obtained from each subject before participating in the study, in accordance with the Declaration of Helsinki. For children aged <18 years, verbal consent was obtained and parents provided written informed consent. All subjects were European Caucasians.

The 97th BMI percentile was used as the threshold for childhood obesity, and children with a BMI lower than the 90th percentile were classified as lean, according to the recommendations of the European Childhood Obesity Group study (26). To calculate the BMI *z* score and threshold for childhood obesity, we used the national growth charts provided by Rolland-Cachera et al. (27) (French children) or Roelants et al. (28) (Belgian children). Adult subjects were defined as follows: lean (BMI <25 kg/m^2), class I obese (BMI ≥30 and <35 kg/m^2), class II obese (BMI ≥35 and <40 kg/m^2), or class III obese (BMI ≥40 kg/m^2).

Screening sample. The populations involved in screening are described in Table 1. The *MC4R* gene was previously sequenced in this sample, and carriers of deleterious mutations were excluded from the screening sample. A set of 422 French class III obese adults and 124 French obese children (BMI *z* score ≥2.35) were first screened for rare exonic *PCSK1* gene mutations. In total, 48 of the 422 French adults were selected from the ABOS (Atlas Biologique de l'Obesité Sévère) cohort and were recruited by the Department of General and Endocrine Surgery, CHRU Lille (29). The other obese French adults and children were recruited by the CNRS UMR8090 and the Department of Nutrition of Paris Hotel Dieu Hospital. Finally, 299 extremely obese adults (BMI ≥50 kg/m^2) were sequenced. This set of 299 patients included 64 obese Belgian patients recruited from the outpatient obesity clinic at the University of Antwerp Hospital (30) and 235 obese Swiss subjects who were recruited for gastric surgery in Zurich, Switzerland (31). To compare the *PCSK1* sequencing data collected in obese subjects with those available in European general populations, we used the May 2011–released sequences from the 1000 Genomes Project (http://www.1000genomes.org) and dbSNP (SNP database) build 131 (http://www.ncbi.nlm.nih.gov/).

Genotyping sample. To find additional carriers of *PCSK1* mutations, 6,233 unrelated obese subjects (BMI ≥30 kg/m^2) and 6,274 lean control subjects (BMI <25 kg/m^2) of European descent were genotyped for eight pathogenic mutations (K26E, M125I, T175M, N180S, Y181H, G226R, S325N, and T558A). Genotyped populations are described in Table 2.

The set of obese subjects included

- 586 unrelated obese French children recruited through a multimedia campaign (CNRS UMR8090)
- 79 obese French children, patients of Toulouse Children's Hospital
- 861 obese French adults recruited by the CNRS UMR8090 and the Department of Nutrition of Paris Hotel Dieu Hospital
- 173 obese Finnish adolescents from the Northern Finland Birth Cohort (NFBC) 1986 (32)
- 1,662 obese Swiss subjects recruited for obesity surgery (Lindberg Clinic) (31)
- 376 obese Belgian children recruited in the Virga Jesse hospital (Hasselt, Belgium) (33)
- 2,496 obese Belgian adults recruited from the outpatient obesity clinic (University of Antwerp Hospital) (30)

The set of control subjects included

- 1,406 lean French subjects selected from the D.E.S.I.R. (Data from an Epidemiological Study on the Insulin Resistance Syndrome) general prospective study (34)
- 4,714 lean Finnish adolescents from the NFBC 1986 (32)
- 154 lean Belgian subjects from University of Antwerp Hospital (30)

Measurements. Weight and height were measured by trained personnel, and BMI was calculated as weight/height^2 (kg/m^2).

Sequencing. Direct sequencing was performed to screen the coding sequence of the *PCSK1* gene. The protocol was carried out using the automated ABI Prism 3730xl DNA sequencer in combination with the BigDye Terminator Cycle Sequencing Ready Reaction kit 3.1 (Applied Biosystems, Foster City, CA). PCR conditions and primer sequences are available on request.

Genotyping. Eight pathogenic mutations were genotyped in a large case-control sample of 6,233 obese and 6,274 lean Europeans. The three mutations located in exon 4 (T175M, Y181H, and N180S) were genotyped by direct sequencing. The M125I and S325N mutations were genotyped using LightCycler 480 High Resolution Melting Master kit (Roche, Basel, Switzerland). Finally, because common variants were located in the vicinity of the G226R and T558A mutations, making the high resolution melting method inefficient, these two mutations were genotyped by melting curve using labeled probes (TIB MOLBIOL, Berlin, Germany) in combination with LightCycler 480. Each mutation detected using the LightCycler 480 was confirmed by direct sequencing. Genotype distribution for the mutations did not deviate from the Hardy-Weinberg equilibrium ($P > 0.05$; analyses made separately in case and control subjects).

In silico analysis. Phylogenetic conservation of the different parts of *PCSK1* containing a mutation was tested using University of California–Santa Cruz Vertebrate Multiz Alignment & Conservation, based on a phylogenetic hidden Markov model, phastCons (35). The impact of the mutations on the three-dimensional structure of PC1/3 was analyzed using the homology model based on the crystal structure of furin and kexin (8,9).

Construction of vector-expressing mutant PC1/3. The expression vector for human PC1/3 containing a FLAG epitope tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) between the propeptide and the catalytic domain has been described previously (6,11). The mutations (K26E, M125I, T175M, N180S, Y181H, G226R, S325N, and T558A) were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, Foster City, CA).

Transient expression of recombinant PC1/3 in cell lines. The neuroblastoma cell line Neuro-2a (N_2A) was transfected with empty vector; or vectors containing cDNAs encoding wild-type PC1/3; or the PC1/3 variants K26E, M125I, T175M, N180S, Y181H, G226R, S325N, T558A, or G593R using Lipofectamine 2000 (Invitrogen, Madison, WI). The human embryonic kidney cell line HEK293T was transfected using FuGENE 6 (Roche, Basel, Switzerland).

PC1/3 maturation and activity. Western blot analyses were performed as described previously using anti–FLAG M2 (Sigma-Aldrich, St. Louis, MO) (6,11). Catalytic activity of the PC1/3 was analyzed using immunopurified enzyme and the fluorogenic substrate pyr-Glu-Arg-Thr-Lys-Arg-amino methylcoumarin (Bachem, Bubendorf, Switzerland) (6,11). Activity was normalized for PC1/3 protein levels as reported previously (15).

Endoglycosidase-F digestion. Deglycosylation experiments were performed essentially as described previously (36). HEK293T cells were transfected with wild-type PC1/3 and T175M, pulse labeled with translabel for 30 min, and chased for 60 min. Cell extracts and medium were immunoprecipitated using M2 antibody, and 50% of these immunoprecipitates were incubated in the absence (−E) or presence (+E) of endoglycosidase-F (Roche) at 37°C overnight. The digestion products and controls were separated by SDS-PAGE.

Statistical analysis. In vitro data are expressed as mean values. Differences between groups were compared with the use of unpaired Student *t* tests (for the activity of PC1/3 recombinants). Logistic regression tests were used to calculate the association of the overall load of deleterious mutations or of the Y118H mutation with obesity, adjusting for age, sex, or geographical origin.

TABLE 1
Clinical characteristics of the sequenced populations

Screening sample of extremely obese patients	*N*	Sex ratio (male to female)	Age at examination (years)	BMI (kg/m^2)	*z*BMI (kg/m^2)
Obese French adults	422	107:315	46.46 ± 12.50	49.16 ± 9.96	—
Obese French children	124	58:66	10.49 ± 3.62	31.29 ± 4.53	8.62 ± 3.29
Obese Swiss adults	235	60:175	42.88 ± 11.22	53.6 ± 3.12	—
Obese Belgian adults	64	27:37	44.76 ± 11.41	54.77 ± 4.98	—

Data are means ± SD.

TABLE 2
Clinical characteristics of the genotyped populations

Case-control study subjects	*N*	Sex ratio (male-to-female)	Age at examination (years)	BMI (kg/m^2)	*z*BMI (kg/m^2)
Obese French children	665	291:374	10.34 ± 3.67	29.03 ± 6.59	6.55 ± 2.91
Obese French adults	861	211:650	46.75 ± 12.80	44.07 ± 6.85	—
Obese Swiss adults	1,662	407:1,255	42.58 ± 11.13	42.24 ± 5.50	—
Obese Finnish children	173	99:74	16	32.47 ± 3.12	3.48 ± 1.04
Obese Belgian children	376	155:221	12.0 ± 3.9	31.2 ± 6.0	2.6 ± 0.5
Obese Belgian adults	2,496	703:1,793	43.90 ± 13.81	36.96 ± 5.03	—
Lean French adults	1,406	478:928	45.87 ± 9.77	21.59 ± 1.7	—
Lean Finnish children	4,714	2,323:2,391	16	20.34 ± 2.61	−0.25 ± 0.62
Lean Belgian adults	154	25:129	34.0 ± 13.0	22.10 ± 3.10	—

Data are means ± SD. Obese adults: BMI ≥30 kg/m^2; obese children: BMI ≥97th percentile for sex and age; lean adults: BMI <25 kg/m^2; and lean children: BMI <90th percentile for sex and age.

A categorical variable was defined to provide information about the geographical origin of the subjects under study: Finland, 1; Belgium, 2; France, 3; and Switzerland, 4. We used a second method, the kernel-based adaptive cluster (KBAC), to assess the association between the overall load of rare variants in *PCSK1* and obesity (37). We performed 200,000 permutations to estimate the KBAC test–derived *P* value of association with obesity. SPSS 14.0 software was used for general statistical analyses (SPSS Inc., Chicago, IL). All reported *P* values are two-sided. $P < 0.05$ was considered significant.

RESULTS

Detection of eight novel mutations in *PCSK1*. The clinical characteristics of the 845 nonconsanguineous extremely obese European subjects are reported in Table 1. Rare variants (frequency <1%) found in the coding sequence were considered a mutation. Eight rare nonsynonymous mutations were identified in eight different carriers (0.95% of the 845 case subjects): the K26E, M125I, T175M, N180S, Y181H, G226R, S325N, and T558A amino acid substitutions. All carriers were heterozygous. The clinical characteristics of each *PCSK1* mutation carrier are reported in Supplementary Table 1. Among the eight *PCSK1* mutation carriers, two subjects were also heterozygous for the two common polymorphisms (Q665E-S690T) that have previously been reported not to significantly impair PC1/3 function (15). All results, including missense, synonymous mutations, and frequent variants (minor allele frequency >5%) are presented in Table 3. To compare these data with those available in European general populations, we used the complete genomic sequences currently available through the 1000 Genomes Project (http://www.1000genomes.org) and through dbSNP build 131 (http://www.ncbi.nlm.nih.gov/). None of the eight mutations and no additional rare mutations were found in these databases.

In silico analysis predicts novel functional mutations. K26 is located one amino acid before the signal peptide cleavage site, and mutation into Glu is predicted to have no effect (http://www.cbs.dtu.dk/services/SignalP/). The M125I, T175M, N180S, Y181H, G226R, and S325N mutations are located in the catalytic domain, and the T558A substitution is located in the middle domain (Fig. 1*A*) (12). Using in silico analysis, a high evolutionary conservation among species was observed for the M125, T175, N180, Y181, G226, S325, and T558 amino acids in 44 vertebrates, including mammalian, amphibian, bird, and fish species (35). In contrast, K26 is weakly conserved. On the basis of the homology model of PC1/3 (9), the mutations in the catalytic and middle domain were analyzed (Fig. 1*B*). M125 is predicted to be located within 8 Å of the Ca-1 site. M125I mutation will result in loss of hydrophobic contacts, potentially affecting the formation of the Ca-1 site after cleavage of the propeptide. T175 also lies within 8 Å of the Ca-1 site, and replacement by Met forces a hydrophobic amino acid to be located at the surface of the protein,

TABLE 3
List of variants identified in *PCSK1* gene

SNP description	dbSNP	Chromosome position	Gene position (exon)	Nucleic acid base change	Amino acid change	MAF
K26E	—	95,794,427	1	a>g	K/E	<0.01
P40P	—	95,794,383	1	c>t	—	<0.01
L90 L	—	95,790,688	2	a>g	—	<0.01
M125I	—	95,787,301	3	g>a	M/I	<0.01
T175M	—	95,784,792	4	c>t	T/M	<0.01
N180S	—	95,784,777	4	a>g	N/S	<0.01
Y181H	—	95,784,775	4	t>c	Y/H	<0.01
N204N	rs6231	95,783,348	5	c>t	N/N	< 0.01
N221D	rs6232	95,777,541	6	a>g	N/D	0.05
G226R	—	95,777,526	6	g>a	G/R	<0.01
S325N	—	95,772,355	8	g>a	S/N	<0.01
N550N	rs6233	95,758,868	12	c>t	N/N	0.38
T558A	—	95,758,846	12	a>g	T/A	<0.01
Q665E	rs6234	95,754,730	14	c>g	Q/E	0.28
S690T	rs6235	95,754,654	14	c>g	S/T	0.28

MAF, minor allele frequency.

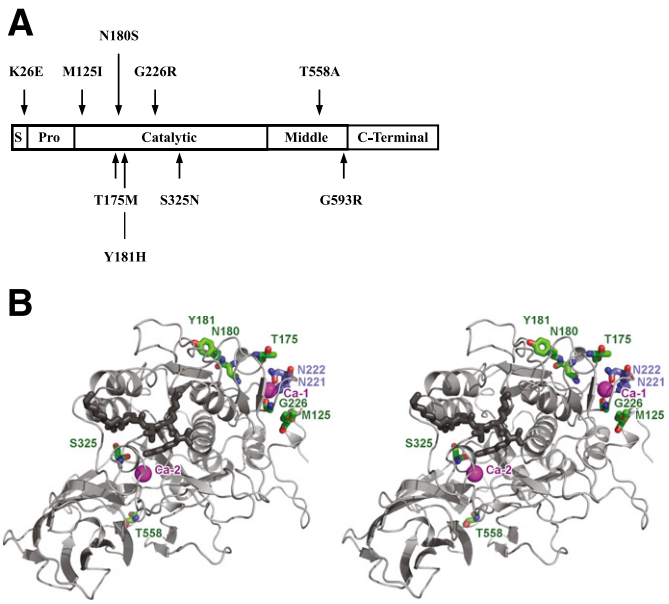

FIG. 1. Location of rare nonsynonymous mutations identified in the screening of 845 extremely obese subjects on the PC1/3 protein. *A*: The domains are depicted in the schematic representation of PC1/3. S, signal peptide; Pro, propeptide; C-Terminal, COOH-terminal domain. G593R has previously been described (12) and is included in this study as a control. *B*: Stereo representation of the PC1/3 model showing the protein backbone in gray cartoon representation, the dec-RVKR-CMK inhibitor in dark gray marking the active site cleft, and the two calcium ions in cyan. The sites of mutations found in this study are represented in green, and the previously described common variant N221D (15) and the N222D mutation found in an obese mouse model (14) are indicated in blue (prepared with PYMOL [DeLano Scientific LLC, www.pymol.org]).

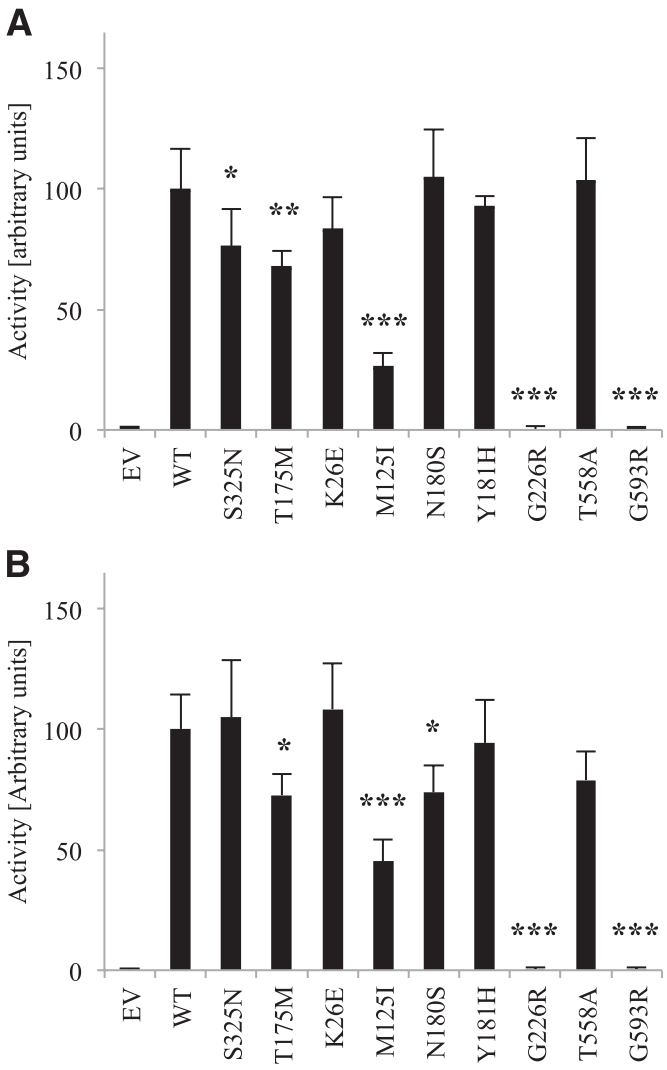

FIG. 2. PC1/3 activity is impaired in G226R, M125I, T175N, and N180S mutants. PC1/3 mutants were immunopurified from conditioned medium of transfected HEK293T and N_2A cells. Activity was determined using the fluorogenic substrate *p*-Glu-Arg-Thr-Lys-Arg-amino methylcoumarin and normalized for the amount of recombinant PC1/3. *A*: N_2A cells. *B*: HEK293T cells. EV, empty vector; WT, wild-type PC1/3. G593R recombinant PC1/3 serves as a negative control. Bars represent mean ± SD; n = 3–6 independent experiments conducted. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

resulting in an energetically unfavorable situation. In addition, T175 is part of the motif for *N*-glycosylation of N173. N180 is also located in close proximity to the Ca-1 site (~11 Å). The shorter side chain of Ser cannot make the stabilizing contacts made by Asn, hence destabilizing the enzyme. The neighboring Y181 is located within 13 Å of the Ca-1 site, and replacement with His is likely to have a similar affect as N180S. G226 is a direct ligand of the Ca-1 site, which contacts the Ca^{2+} via its carbonyl oxygen. Because there is no space for the much larger and conformationally restricted Arg, this mutation is predicted to have a severe impact. The S325N mutation is energetically unfavorable because the Asn requires more space and forms different hydrogen bonds, which will destabilize the enzyme. Finally, the T558A mutation disrupts the specific hydrogen bonds made by the hydroxyl side chain of Thr and the surrounding S555 and T533. This mutation is therefore predicted to destabilize the fold of the middle domain. Taken together, the homology model of PC1/3 indicates that all seven mutations in the catalytic and middle domains will have an impact on the folding and stability of the enzyme, albeit at varying degrees of severity.

Functional characterization confirmed and amplified in silico predictions. The functional consequences of the K26E, M125I, T175M, N180S, Y181H, G226R, S325N, and T558A mutations on PC1/3 maturation and activity were investigated in transiently transfected HEK293T fibroblasts and N_2A neuroectodermal cells. The activity was assessed by measuring the processing of a fluorogenic substrate by recombinant PC1/3 mutants immunoprecipitated from conditioned medium (Fig. 2). As an internal control, the G593R loss-of-function mutation was used (12). In vitro studies confirmed the catalytic inactivity of G593R (99.4% decrease compared with wild type PC1/3; $P < 10^{-5}$) and blocked propeptide cleavage and secretion (Fig. 3), validating the experimental design. A complete loss of catalytic activity was observed for recombinant G226R (99.4% decrease; $P < 10^{-5}$), whereas the M125I mutation induced a significant reduction in activity by 73.6% ($P < 0.01$) and 54.9% ($P < 0.001$) in HEK293T and N_2A cells, respectively. In a similar manner, the activity of T175M was reduced in both cell lines (32.1% in HEK293T, $P < 0.01$; and 27.3% in N_2A cells, $P < 0.05$). N180S had a significantly decreased activity in N_2A cells only (26.3%, $P < 0.05$), consistent with the more pronounced difference in COOH-terminal processing of this mutant in N_2A cells (Fig. 3). S325N had a decreased activity in HEK293T cells only (23.5%, $P < 0.05$). The T558A and the Y181H amino acid changes did not appear to influence the enzymatic activity significantly in either cell line.

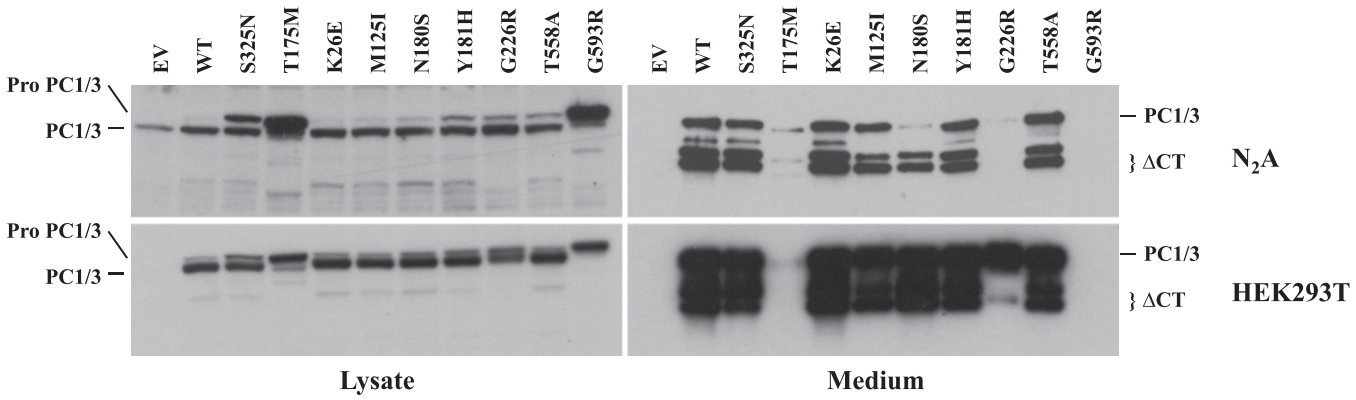

FIG. 3. Maturation and/or secretion are impaired in seven of eight PC1/3 mutants. Western blots of cell lysates and conditioned medium of transfected N_2A and HEK293T cells with EV (empty vector), WT (wild-type PC1/3), S325N, T175M, K26E, M125I, N180S, Y181H, G226R, T558A, or G593R using FLAG M2 for detection of recombinant PC1/3 proteins. ΔCt corresponds to COOH-terminal truncated PC1/3. G593R recombinant PC1/3 serves as a negative control.

The consequences of these mutations on maturation and secretion were characterized by Western blotting (Fig. 3). G226R and T175M showed reduced propeptide cleavage, COOH-terminal processing, and secretion compared with wild-type PC1/3 in both HEK293T and N_2A cells. The S325N, Y181H, and T558A mutations affected only propeptide cleavage, particularly notable in N_2A lysates. It is remarkable that in N_2A cells, the N180S mutation resulted in a severely reduced amount of secreted full-length PC1/3, with little reduction of the COOH-terminally processed forms. No change in maturation or secretion was observed for K26E.

For T175M, a slight downward shift for all bands was observed, most notably in the N_2A medium. The T175M mutation is located at one of the two potential *N*-glycosylation sites of PC1/3. Therefore, the implication on *N*-glycosylation was investigated using the deglycosylation enzyme endoglycosidase-F. No reduction in molecular weight was found after endoglycosidase-F digestion of T175M, either in lysate or in medium, whereas wild-type PC1/3 forms shifted downward to the same apparent molecular weight as the T175M mutant (Fig. 4).

Altogether, these data show that seven of eight mutations have a functional effect on PC1/3: G226R induces complete loss of activity, whereas S325N, M125I, T175M, N180S, Y181H, and T558A mutations are partially deleterious, and only the K26E substitution seems to have no detectable impact. The functional data are summarized in Supplementary Table 2.

Deleterious *PCSK1* mutations associate with common obesity. After genotyping the eight mutations in 6,233 unrelated obese subjects and 6,274 control subjects of European descent (Table 2), 11 additional carriers for the Y181H mutation were identified (9 obese and 2 control subjects) as well as 2 obese subjects for the N180S mutation and 1 obese subject for the M125I mutation. No new carriers for the K26E, T175M, S325N, G226R, or T558A mutations were found. The clinical characteristics of each *PCSK1* mutation carrier in the case-control study are reported in Supplementary Table 3. The putative association of these mutations with obesity was first assessed by comparing the prevalence of the overall load of the deleterious mutations in *PCSK1* in obese and lean subjects using logistic regression adjusted for age, sex, and geography. The load of deleterious mutations in *PCSK1* showed a statistically significant 8.7-fold enrichment in obese subjects compared with lean control subjects (odds ratio [OR] 8.66 [95% CI 1.04–72.01]; $P = 0.046$) (Table 4). Using the recently developed KBAC method to assess the association between the overall load of rare loss-of-function coding variants in *PCSK1* and obesity gave similar results (OR 6.07, $P = 0.006$). This overall association was mainly driven by the more frequent mutation Y181H that showed a borderline association with obesity when analyzed solely in a logistic regression model (7.31 [0.84–63.55]; $P = 0.072$) (Table 4).

DISCUSSION

The most important observation in this study is that rare nonsynonymous mutations causing partial PCSK1 deficiency are enriched in common severe obesity. Furthermore, 0.83% of our cohort of subjects with extreme obesity carry these variants, suggesting that after *MC4R* (38) and the chromosome 16p deletion (39), *PCSK1* is the third most prevalent contributor to extreme obesity in European populations identified so far. We previously reported that the modestly deleterious variant N221D is associated with a small increase in the risk for obesity (15). Not only null mutations cause a recessive monogenic form of obesity with syndromic features (6,11,12); we show here that

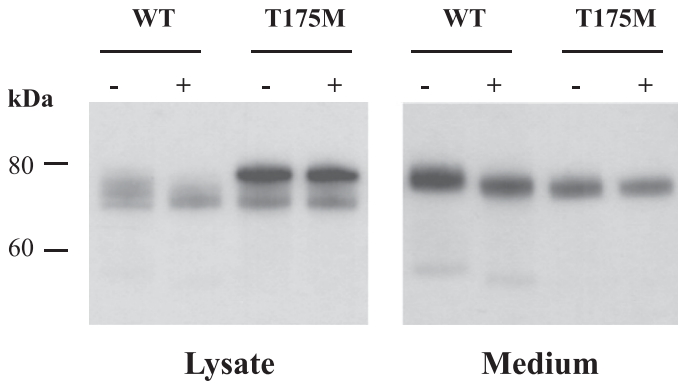

FIG. 4. The T175M mutation impairs *N*-glycosylation of PC1/3. Analysis was performed on transfected HEK293T cells. Absence (−) or presence (+) of endoglycosidase-F. Note the reduction in molecular weight of wild-type (WT) PC1/3 in the medium to the same apparent molecular weight of untreated (and treated) T175M mutant.

TABLE 4
Frequency of deleterious *PCSK1* mutations in obese and lean subjects

Variant	Obese carrier (*n*)	Frequency obese (%)	Lean carrier (*n*)	Frequency lean (%)	OR (95% CI)*	*P* value*
M125I	1	0.02	0	0	—	—
T175M	0	0	0	0	—	—
N180S	2	0.03	0	0	—	—
Y181H	9	0.14	2	0.03	7.3 (0.8–63.5)	0.072
G226R	0	0	0	0	—	—
S325N	0	0	0	0	—	—
T558A	0	0	0	0	—	—
Total	12 of 6,233	0.19	2 of 6,274	0.03	8.7 (1.0–72.0)	0.046

*OR (95% CI) and *P* value of the association with obesity. The logistic regression has been adjusted for age, sex, and geography.

haploinsufficient heterozygous mutations, although infrequent, associate with a rather penetrant form of obesity. Indeed, these functional variants increase the risk of developing obesity 8.7 times in Europeans. Association does not imply causality, but by combining the sequencing data with the comprehensive studies on PC1/3 maturation and activity, the functional molecular link between the *PCSK1* genotype and obesity becomes likely.

Dickson et al. (40) recently proposed that rare genetic variants, by occurring, stochastically, more often in association with one of the alleles at the common site versus the other allele, can create synthetic associations that are credited to common variants. Our data do not favor the synthetic association hypothesis at the *PCSK1* locus and, rather, support an independent contribution of rare and common coding variants in *PCSK1* to obesity predisposition, as recently demonstrated at the *MC4R* locus (41). Among the eight *PCSK1* rare mutation carriers identified in our screening of 845 obese subjects, only 2 subjects are also heterozygous for the common polymorphisms Q665E-S690T, signifying that no N221D carrier and only 0.5% of the Q665E-S690T carriers harbor simultaneously rare deleterious mutations in *PCSK1*.

An important finding is that seven of eight of the novel nonsynonymous *PCSK1* mutations found in the screening of extremely obese patients were deleterious. It shows the important role of this gene in obesity because the average of damaging nonsynonymous mutations for complex diseases in humans has been estimated to be 73% (42). Of the eight mutations we detected in obese subjects, one resulted in a total loss of function, six were partially deleterious, and only the K26E mutation was found neutral by both prediction and functional analysis. A remarkable observation is that five damaging mutations are predicted to affect the Ca-1 binding site. The Ca-1 site is conserved in eukaryotic PCs and bacterial subtilisins. Mutation of this site in subtilisin results in an active enzyme, albeit with significantly lower stability (43). The N222D mutation in the previously reported obese mouse model (14) also affects the Ca-1 site. N222 is one of the side chain ligands of the calcium ion, and although the side chain oxygen of Asp might take over, its charge, polarity, and hydrogen-bonding potential are clearly different. Maturation of N222D is normal except for reduced COOH-terminal processing, and its activity is reduced by 36%. Although this effect on functionality might appear limited, the obese phenotype segregated in a dominant manner in mice fed a high fat diet (14). The frequent variant N221D that increases the risk of obesity can therefore be expected to influence the Ca-1 site by changing the position of N222. The side chain of residue 221 mostly interacts with the solvent, so a major change is not expected. In furin, the corresponding Asn (N207) makes a strong hydrogen bond with the amide nitrogen of a Thr (T114). This might also be the case in PC1/3, but the homology model is not sufficiently accurate to allow this prediction. The change in charge caused by the N221D variant, however, is likely to have at least some effect on this region and the Ca-1 site.

For the first time, a mutation on an *N*-glycosylation site of PC1/3 was identified. *N*-glycosylation is a key process in cellular signaling and protein maturation that starts in the ER and concludes in the Golgi compartment. Our study shows that the T175M mutation in the *N*-glycosylation site NHT induces an inhibition of *N*-glycosylation of PC1/3, indicating that the second potential *N*-glycosylation site NLT at position 401 is not used. This is consistent with a recent study that shows that in mouse PC1/3, which contains three potential *N*-glycosylation sites, the first site is critical for propeptide cleavage, whereas the second is not used (44). The third site, present in mouse but not human PC1/3, is of lesser importance. Our in vitro data confirm this because the T175M mutation altered the maturation and secretion of the protein and reduced the enzymatic activity significantly in both HEK293T and N_2A cells.

The differential effect of the mutations in the two cell lines may be the consequence of different relative expression levels of secreted full-length and COOH-terminal processed PC1/3. COOH-terminal processing of PC1/3 is cell-type dependent and, for instance, is more pronounced in pancreas than in brain. Mutations can have a cell type–dependent effect on COOH-terminal processing of PC1/3, as described before for the N222D mutation (14).

In the three previously reported case subjects harboring complete PC1/3 deficiency, probands were either compound heterozygote (6,12) or homozygote (11). In these studies, the eight heterozygous carriers of mutations in pedigrees were described as clinically unaffected. This result is somewhat surprising because heterozygosity for other monogenic obesity genes, such as *MC4R* (45), *POMC* (46), *LEP* (47), and *LEPR* (48) mutations, are usually associated with an increased body weight. Furthermore, heterozygous N222D mice presented an obesity-intermediate phenotype (14). In the current study, the observation of 21 heterozygous carriers of *PCSK1* deleterious mutations and their association with obesity suggest that partial PC1/3 deficiency contributes to an energy imbalance in humans.

One limitation of this study is that a multicentric recruitment of lean and obese European patients was performed to achieve a large sample size. Case and control subjects were recruited in different European countries (Finland, France, Belgium, and Switzerland) and, therefore, introduced a potential cause of genetic heterogeneity.

Because European populations can be classified into two clusters, the northern (including Finnish, French, Belgian, and Swiss) and the southern (including Greek, Italian, Spanish, and Portuguese) (49), the populations included in this study are part of the same northern European genetic cluster and are less likely to display major genetic differences. This is reinforced by the fact that correcting for geographical area did not affect the significance of the association between the overall load of deleterious nonsynonymous mutations and obesity. However, we acknowledge that geographic location is only a proxy for population substructure, and correcting the data for geographical area does not perfectly account for latent population stratification. Another limitation of this study could be the introduction of a bias concerning the risk ratios reported as a result of the study design (50). Indeed, the recruitment of study case subjects has been performed in clinical centers based on obesity criteria, and obese individuals have not been selected from a population-based study. However, given the rarity of the deleterious mutants, population cohorts are probably not sufficiently enriched in extreme obesity cases to offer adequate statistical power for rare variant analysis in candidate genes. Another limitation of this study relates to the fact that other rare deleterious mutations might be present in the 6,233 obese and 6,274 lean subjects that were not detected by genotyping the seven loss-of-function mutations identified in our screening. The estimation of the prevalence of deleterious *PCSK1* mutations in the obese (0.19%) and lean (0.03%) subjects is therefore likely to underestimate the real prevalence in the case-control study.

In conclusion, our results suggest that rare pathogenic variants in genes involved in appetite regulation, such as *PCSK1*, may contribute to obesity risk. The current and previous studies (12,15) support that a continuous spectrum of genetic defects in *PCSK1* (from the rare coding mutations to the common polymorphisms) contributes to the genetic architecture of obesity, as also observed at the *MC4R* locus (51–53). Although modest at the population level, mutations that may result in partial PCSK1 deficiency should be considered as a serious risk factor for future, extreme obesity at an individual level.

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